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<b>(21) International Application Number:</b> PCT/US91/00213 <b>(22) International Filing Date:</b> 10 January 1991 (10.01.91)  <b>(30) Priority data:</b> 463,022 10 January 1990 (10.01.90) US  <b>(71) Applicant:</b> CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US). <b>(72) Inventor:</b> URDEA, Michael, S. ; 100 Bunce Meadow Road, Alamo, CA 94507 (US). <b>(74) Agents:</b> DOLBERG, David, S. et al.; Irell & Manella, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025 (US).		<b>(81) Designated States:</b> AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> DNA-DEPENDENT RNA POLYMERASE TRANSCRIPTS AS REPORTER MOLECULES FOR SIGNAL AMPLIFICATION IN NUCLEIC ACID HYBRIDIZATION ASSAYS  <b>(57) Abstract</b> <p>A polydeoxynucleotide construct is disclosed for use, in conjunction with a DNA-dependent RNA polymerase, as a signal amplifier in nucleic acid hybridization assays. The construct contains a recognition sequence for a target oligonucleotide, a promoter sequence for a DNA-dependent RNA polymerase, and a polymerase template. A method of use for this construct in hybridization assays is also disclosed. The method involves formation of a hybridization complex comprising the construct and the target sequence; addition of a polymerase which is specific for the promoter in the construct; and quantification of the resulting RNA transcripts.</p>		

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5 "DNA-Dependent RNA Polymerase Transcripts as Reporter Molecules  
for Signal Amplification in Nucleic Acid Hybridization Assays".

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#### Background of the Invention

##### Technical Field

This invention pertains to the detection and  
quantification of biomolecules by hybridization assay,  
20 and pertains more particularly to hybridization assays  
wherein reporter molecules are used for signal  
amplification.

##### Background

25 Nucleic acid hybridizations are now commonly  
used in genetic research, biomedical research and  
clinical diagnostics to detect and quantify particular  
nucleotide sequences which are present in heterogeneous  
mixtures of DNA, RNA, and/or other materials. In the  
30 basic nucleic acid hybridization assay, single-stranded  
analyte nucleic acid (either DNA or RNA) is hybridized,  
directly or indirectly, to a labeled nucleic acid probe,  
and the duplexes containing label are quantified. Both  
radioactive and n nradioactive labels have been used.

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The basic assay lacks sensitivity. When the analyte is present in low copy number or dilute concentration the signal cannot be distinguished from the background noise. Variations of the basic scheme have been developed to facilitate separation of the target duplexes from extraneous material and/or to amplify the analyte sequences in order to facilitate detection, but these variations have suffered generally from complex and time consuming procedures, high background, low sensitivity, and difficulty in quantification. A primary object of the present invention is to provide an amplifier for use in hybridization assays that provides a highly reproducible gain in signal, a highly reproducible signal-to-noise ratio, is itself quantifiable and reproducible, and is capable of combining specifically with an analyte present at low concentration and with a "universal" reporter moiety to form a stable complex.

Commonly owned US Patent No. 4,868,105, issued 19 September 1989, the disclosure of which is hereby incorporated by reference, describes a solution phase hybridization sandwich assay in which the analyte nucleic acid is hybridized to a "labeling probe" and to a "capturing probe". The probe-analyte complex is coupled by hybridization to a solid-support. This permits the analyte oligonucleotide to be removed from solution as a solid phase complex, thereby concentrating the analyte, facilitating its separation from other reagents, and enhancing its subsequent detection.

PCT Application 84/03520 and EPA 124221 describe a DNA hybridization assay in which: (1) analyte is annealed to a single-stranded DNA probe that has a tail that is complementary to an enzyme-labeled oligonucleotide, and (2) the resulting tailed duplex is hybridized to an enzyme-labeled oligonucleotide. The Enzo Biochem "Bio-Bridge" labeling system appears to be

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similar to the system described in these two patent applications. The "Bio-Bridge" system uses terminal deoxynucleotide transferase to add unmodified 3'-poly T-tails to a DNA probe. The poly T-tailed probe is  
5 hybridized to the target DNA sequence and then to a biotin-modified poly A.

EPA 204510 describes a DNA hybridization assay in which analyte DNA is contacted with a probe that has a tail, such as a poly dT-tail, and an amplifier strand  
10 that has a sequence, e.g., a poly dA sequence, that hybridizes to the tail of the probe and is capable of binding a plurality of labeled strands.

The main problem with these prior hybridization assays is that they lack sufficient specificity and/or  
15 signal to be useful for detecting very low levels of analyte.

Another commonly owned EP Application No. 88309697.6 (publication No. 0317077), filed 17 October 1988, the disclosure of which is hereby incorporated by  
20 reference, describes linear and branched oligonucleotides which can be used as a signal amplifiers in hybridization assays. Here the amplifier oligomer has two domains--a first domain which is complementary to a target sequence (either the analyte per se or a "linker probe") and a  
25 second domain, present in repeating units, complementary to a labeled reporting sequence. The multiplication of reporting sequences per target sequence provides for the amplification of the signal.

Another approach has been to use nucleic acid  
30 polymerases to amplify target sequences. For example, the so-called polymerase chain reaction (PCR), uses repeated cycles of DNA primed, DNA-directed DNA polymerase synthesis to amplify sequences of interest  
(Saiki, R.K., et al., Science (1986) 230:1350-1354). The

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amplified target is then detected using the basic hybridization assay protocol.

RNA polymerases have also been used to amplify target sequences (Krupp, G., and Soll, D. FEBS Letters (1987) 212:271-275). This approach has been incorporated into a hybridization format that involves production of a double-stranded copy of the target sequence, insertion of a RNA polymerase promoter sequence, transcription of the copy and detection by hybridization assay (Kwoh, D.Y., et al., Proc. Natl. Acad. Sci. U.S.A. (1989) 86:1173-1177). Since DNA-directed RNA polymerases produce up to 10<sup>3</sup> copies of RNA per copy of DNA template, fewer cycles of amplification are required. Bacteriophage DNA-dependent RNA polymerases (e.g., T3, T7, SP6) have previously been employed for the preparation in vitro of specific RNA sequences from cloned or synthetic oligonucleotide templates and are well understood (Melton, D.A., et al., Nucleic Acids Res. (1984) 12:7035-7056); Chamberlin, M. and Ryan, T., (1982) in "The Enzymes," Boyer, P.D., ed., 15:87-108; Martin, C.T., and Coleman, J.E., Biochemistry (1987) 26:2690-2696). These polymerases are highly promoter specific. DNA sequences from 17 T7 promoters are known and a consensus sequence has been deduced (Oakley, J.L., and Coleman, J.E., Proc. Natl. Acad. Sci. U.S.A. (1977) 74:4266-4270; Dunn, J.J., and Studier, F.W., J. Molec. Biol. (1983) 166:477-535). It is also known that to retain polymerase activity, only the promoter region must be double-stranded (Milligan, J.F., et al., Nucleic Acids Res. (1987) 15:8783-8799).

Finally, RNA-directed RNA polymerase has also been used to detect target sequences (Lizardi, P.M., et al., Bio/technology (1988) 6:1197-1202; Lomeli, H., et al., Clin. Chem. (1989) 35:1826-1831). In this system an RNA probe is prepared by coupling RNA complementary to the target sequence with RNA (MDV-1) (U.S. Patent No.

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4,786,600) which serves as an exclusive template for the bacteriophage Q-beta (Q) replicase. First, the target is immobilized on a solid substrate, then the RNA probe is hybridized to the target and finally the probe is eluted.

5 Subsequent addition of Q-beta-polymerase to the probe generates multiple copies of the template/target RNA. In a related assay, MDV-1 RNA was first bound to biotin, then coupled to an avidinylated target, and subsequently assayed as described above (Chu, B.C.F., et al., Nucleic

10 Acids Res. (1986) 14:5591-5603).

The use of Q-beta-replicase in hybridization assays has four major disadvantages:

1) Q-beta-replicase is typically contaminated with MDV-1 RNA. Consequently, this system has very high

15 background (poor signal-to-noise ratio) when the reporter sequence is the MDV-1 sequence itself;

2) The probe is RNA. RNA is highly sensitive to degradation from the RNAase activity which is ubiquitous in crude cellular preparations, and from the

20 alkaline conditions required to denature double-stranded DNA targets;

3) Due to the secondary structure of MDV-1 RNA there is considerable nonspecific binding in hybridization assays, thus significantly lowering the sensitivity

25 of the assay and precluding accurate quantification; and,

4) The amount of signal (the RNA product of Q-beta-replicase) varies with the log of the number of probes originally bound to the target. Thus, this assay can only detect order-of-magnitude differences between

30 the concentrations of analyte in various samples.

The invention disclosed herein has several advantages over the Q-beta-replicase method. First, the probe is DNA rather than RNA. Second, the assay has very high signal to noise ratio and very high sensitivity.

35 Third, since the signal is amplified rather than the

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target, the oligom r which is actually measured will always have the same sequence and size, thereby enabling the standardization and optimization of assay conditions (in addition, most of the biological reagents can be used 5 universally thereby further simplifying and standardizing the assay). Finally, the target can be easily and accurately quantified.

#### Summary of the Invention

10 One aspect of the invention is a polydeoxynucleotide construct (template probe) for use as a signal amplifier in hybridization assays. The template probe is comprised of three domains as depicted in Figure 1A:

(i) a first domain (A) which is single-stranded 15 and has a nucleotide sequence (a') complementary to a target sequence (a) (Figure 2A) the target sequence comprising a domain either within the analyte sequence or within the sequence of an oligonucleotide which also contains a sequence domain complementary to the analyte 20 sequence;

(ii) a second domain (B) which is double-stranded and capable of function as a promoter for a DNA-dependent RNA polymerase enzyme activity; and

(iii) a third domain (C) which is either 25 single- or double-stranded and adjacent to the second domain, such that the third domain is capable of functioning as a template (c') for the promoter activity of the second domain (Figure 2B).

A second aspect of the invention is a method of 30 amplifying the biological signal used to detect and quantify an oligonucleotide, or other biomolecular analyte, in a hybridization assay comprising the following steps:

(i) immobilizing the analyte, directly or 35 indirectly, on a solid substrate; and hybridizing the



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polydeoxynucleotide template probe described supra,  
directly or indirectly, to the analyte;

(ii) next removing the unhybridized template  
probe;

5 (iii) next transcribing (via a DNA-dependent  
RNA polymerase activity) multiple copies of RNA oligomers  
(c) which are complementary to the template sequence (c')  
of domain C of the amplifier; and

(iv) finally quantifying the RNA transcripts.

10

#### Brief Description of the Drawings

Figure 1A is a schematic representation of a  
monomeric template probe. Capital letters designate  
domains, and lower case letters designate strands within  
15 a domain. A primed letter designates a lower strand  
(read 3'- to 5'-, left to right). The a' sequence is  
complementary to a target sequence. The B domain is the  
promoter for a RNA polymerase. The c' sequence is the  
template for the RNA polymerase. The probe is  
20 synthesized as a single strand. The AAAAAAA represents  
the poly-A linker added to allow for self-annealing.

Figure 1B is the DNA sequence of one embodiment  
of the template probe. The promoter domain, B, consists  
of the consensus sequence of the bacteriophage T7  
25 promoter (5'-TAATACGACTCACTATA-3') plus 15 additional  
residues 5' to the promoter sequence.

Figure 1C is a schematic representation of a  
multimeric template probe in which the double-stranded  
regions are self-annealing.

30 Figure 2A is a schematic representation of a  
sandwich hybridization assay system which incorporates  
the template probe. The analyte is indirectly  
immobilized upon a solid substrate by hybridization to  
the analyte capture probe and indirectly joined to the  
35 template probe via the template linker probe. "w"

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represents the sequence of a region of the immobilized polynucleotide which is complementary to a region (w) of the analyte capture probe. "y" represents the sequence of a region of the analyte capture probe which is complementary to a region (y') of the analyte. "x'" represents the sequence of a region of the analyte which is complementary to a region (x) of the template linker probe. "a" represents a sequence of the template linker probe which is complementary to a', the sequence of the A domain of the template probe.

Figure 2B is a schematic representation of the use of RNA polymerase transcripts as reporter molecules in a hybridization assay. After hybridization of the analyte and template probe, an RNA polymerase is added and multiple RNA transcripts complementary to the template sequences (c) are produced. These sequences have two sub-domains:  $c_1$  which is complementary to a capture probe immobilized upon a solid substrate; and  $c_2$  which is complementary to a labeling probe. This allows for indirect immobilization of the label and easy quantification of the hybridization assay signal. "\*" designates the incorporated label which may be radioactive, chemiluminescent, fluorescent or enzymatic.

Figure 2C is the DNA sequence of a transcript of the C domain, as well as the sequence of the transcript capture probe ( $C_1'$ ), and the labeling probe ( $C_2'$ ). X represents the N<sup>4</sup> methyl deoxycytidine derivative, [(6-aminocaproyl)-2-aminoethyl]-5-methyl-2'-deoxycytidine, used to couple the capture probe to the solid phase.

Figure 3A depicts the preparation of pII template probe.

Figure 3B depicts the preparation of pII<sub>L</sub> template probe.

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Figure 3C depicts the preparation of pII<sub>R</sub> template probe.

Figure 4 depicts the domain A oligomer of Example 9 and the DNA sequences of oligo N, oligo S and  
5 oligo H.

Figure 5 depicts a protocol for a nucleic acid assay utilizing the T7 template probe and also utilizing an amplifier probe to further increase sensitivity and amplification of the signal.

10 Figure 6 is a graph showing the relative sensitivity and signal amplification of various template probes in assays for the presence of HIV in human serum.

#### Detailed Description of the Invention

15 A "biological signal" is a biochemically transmitted indicium of the occurrence of an event or presence of a specific molecule.

"DNA-dependent RNA polymerase" is an enzyme which facilitates the polymerization of RNA of specific  
20 sequence from a complementary DNA template.

A "domain" is a particular region of a polynucleotide characterized by its function.

An "immunological reaction" is the specific recognition and binding of an antibody to its correspond-  
25 ing epitope.

A "polydeoxynucleotide" is a polymeric DNA molecule. A "polynucleotide" is a polymeric DNA or RNA molecule.

A "promoter" is the site on a polydeoxy-  
30 nucleotide to which a RNA polymerase enzyme binds preparatory to initiating transcription.

"RNA-dependent RNA polymerase" is an enzyme which facilitates the polymerization of RNA of specific sequence from a complementary RNA template.

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"Transcription" is a process, mediated by an enzyme, by which RNA is formed corresponding to a complementary polynucleotide template.

5 The "upper strand" of a double-stranded DNA molecule is the strand whose 5'-end is on the left as the sequence is read from left to right. The sequence of this strand is always presented above the sequence for its complementary "lower strand" which is read 3'- to 5'-, left to right.

10

#### Modes for Carrying Out the Invention

##### 1. Template Probe

In one aspect of this invention a DNA probe (referred to as a "template probe") containing three  
15 functional domains has been designed in order to enhance the signal in hybridization assays.

The first domain ("A" in Figure 1A), has a sequence (a') usually 10 to 40 nucleotides in length, preferably 15 to 30 nucleotides, is single-stranded and  
20 is designed to hybridize to a complementary target sequence (a). In order to achieve hybrid stability, this domain will normally have a GC content in the range of 40% to 60%. The target sequence may subsist within the overall sequence of the polynucleotide to be assayed  
25 (referred to as the analyte) or it may subsist within an oligonucleotide linker which also has homology to the analyte. In a preferred embodiment, the analyte will be immobilized upon a solid substrate to facilitate subsequent washing procedures. This immobilization may  
30 be direct (e.g., polynucleotide preparations containing the analyte might be bound to a nitrocellulose filter) or indirect (e.g., a linker might be immobilized on the filter and the analyte subsequently hybridized to the linker).

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The second domain (B), usually 10 to 40 basepairs in length, preferably 20 to 35 nucleotides, more preferably 30 to 35 nucleotides, is double-stranded and functions as a DNA-directed RNA polymerase promoter.

5 This promoter is usually derived from the promoter sequence of a bacteriophage, preferably any of the phage T3, T7, or SP6, more preferably from bacteriophage T7. This class of RNA polymerases is highly promoter specific. The T7 promoter is probably the best  
10 characterized. DNA sequences from 17 T7 promoters are known and a consensus sequence had been deduced: 5'-TAATACGACTCACTATA-3' (Oakley and Coleman; Dunn and Studier). Sequences 3' to the promoter on the  
15 complementary strand (the c' segment, whose 3' end is adjacent to the 5' end of the b' segment) serve as the template for transcription and the transcription of many template sequence variations can be accommodated. Only the promoter region itself must be double-stranded (Milligan et al.).

20 Additional sequences may be added at the 5' end of the promoter. For example, in a preferred embodiment, the B region consists of the consensus sequence of the T7 promoter plus additional bases 5' to the consensus sequence which are identical to the sequence of the pT7  
25 plasmids (available from US Biochemicals) up to the PvuII restriction site (Figure 1B). These sequences may or may not have an effect on transcription.

The third domain (C) is directly 3' to the second domain and the c' strand of this domain serves as  
30 the template for the domain B promoter. Domain C may be as small as 30 nucleotides in length, or as long as 10 Kb. In a preferred embodiment the domain is 40 to 45 bases. In another preferred embodiment the domain is 3.4 Kb and is substantially similar to the genomic DNA of  
35 Hepatitis B virus. This domain may be either single- or

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double-stranded, and the 3' end of the c' template strand (directly adjacent to the promoter) usually is a cytosine residue.

5       The proper 5' to 3' relation of the promoter (B domain) to the template (C domain) is necessary for proper transcription of the template. The promoter is directly 5' to the template and the template is read 3' to 5'. However, it will be appreciated by those skilled in the art that the orientation of the B/C domains to the  
10   A domain is not critical. Thus template probes constructed as domains A-B-C, or as B-C-A will produce the same transcript and therefore may be constructed in either form.

15       The RNA transcription product (c) of the C domain functions as a reporter molecule for the presence and quantity of analyte. Signal amplification occurs because each template produces  $10^1$  to  $10^4$  transcripts. The sequence of this domain may be designed with a random sequence, evaluated by computer analysis to minimize the  
20   possibility of cross-reaction with other probes in the system, or alternatively, may be a known sequence which has been specifically chosen.

      Further amplification can be achieved by designing the template probe with multimeric  
25   promoter/template (B/C) domains (Figure 1C). These multimeric units may be either in a linear array or branched molecules. For further details concerning the technology for the production and application of such multimers in hybridization assays, see EPA publication  
30   No. 0317077.

      In a multimeric template probe the total number or repeating B/C units will usually be in the range of 2 to 200, more usually 5 to 20. The B/C units of the multimer may be covalently linked directly to each other  
35   through phosphodiester bonds or through interposed

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linking agents such as nucleic acid, amino acid, carbohydrate or polyol bridges, or through other cross-linking agents that are capable of cross-linking nucleic acid strands. The site(s) of linkage may be at the ends of the unit (in either normal 3'-5' orientation or randomly) and/or at one or more internal nucleotides in the strand. In linear multimers the individual units are linked end-to-end to form a linear polymer. In branched multimers three or more oligonucleotide units emanate from a point of origin to form a branched structure. The point of origin may be another oligonucleotide unit or a multifunctional molecule to which at least three units can be covalently bound. The multimer may be totally linear, totally branched, or a combination of linear and branched portions. Preferably there will be at least two branch points in the multimer, more preferably at least three. The multimer may include one or more segments of double-stranded sequences.

Template probes may be prepared by cloning, enzymatic assembly, chemical cross-linking techniques, direct chemical synthesis or a combination thereof. When prepared by cloning, nucleic acid sequences that encode the entire probe or fragments thereof can be made in single- or double-stranded form by conventional cloning procedures. When made in double-stranded form, the probe is denatured to provide single strands. Template probes may also be cloned in single-stranded form using conventional single-stranded phage vectors such as M13.

The A domain is single-stranded, the B domain is double-stranded, and the C domain may be either single- or double-stranded. A particular domain (e.g., B domain) can subsequently be made double-stranded by hybridization with its complementary strand--cloned separately. Alternatively, the entire template prob can be cloned as a singl -stranded, self-annealing

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p lyncleotide (a' b' c' c b). In this case four to ten additional nucleotides, preferably 5-7 nucleotides, are added to the sequence as a spacer between c and c' to allow for proper contouring of the double-stranded region when it is self-annealed. The spacer is usually poly-A, but may be modified to minimize hybridization cross-reactivity between various probes in the assay.

If multimeric probes are desired, fragments are linked enzymatically or chemically to form the multimer. When assembled enzymatically, the individual units are ligated with a ligase such as T4 DNA ligase. When prepared by chemical cross-linking, the individual units may be synthesized with one or more nucleic acids that have been derivatized to have functional groups that provide linking sites or derivatized after the oligonucleotide has been synthesized to provide such sites. A preferred procedure for chemical cross-linking is to incorporate N<sup>4</sup>-modified cytosine bases into the nucleotide as described in the commonly owned EPA publication No. 0225807.

When prepared by direct chemical synthesis oligonucleotides containing derivatized nucleic acids whose functional groups are blocked are made by conventional oligonucleotide synthesis techniques. The functional groups are unblocked and oligonucleotide units are synthesized out from the unblocked site(s).

## 2. Amplified Hybridization Assay

Another aspect of this invention employs template probes in hybridization assays. The analyte may be any nucleotide sequence of interest--either DNA or RNA. The analyte sequence may constitute an entire molecule or only a portion of a molecule. The analyte may be a homogeneous polynucleotide, present in low concentration in a prepared sample or it may be a



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minority species in a heterogeneous mixture of polynucleotides. The analyte may also be from a variety of sources, e.g., biological fluids or tissues, food stuffs, environmental materials, etc., or it may be synthesized in vitro.

The analyte may be prepared for the hybridization analysis by a variety of means, e.g., proteinase K/SDS, chaotropic salts, etc. Also, it may be of advantage to decrease the average size of the analyte by enzymatic, physical or chemical means, e.g., restriction enzymes, sonication, chemical degradation (e.g., metal ions), etc. The fragments may be as small as 0.1 kb, usually being at least about 0.5 kb and may be 1 kb or higher. Where the analyte sequence is lengthy, for example a viral genome, several different regions of the analyte may be used as targets of an analyte probe.

The analyte sequence is provided in single-stranded form for analysis. Where the sequence is naturally present in single-stranded form, denaturation will not usually be required. However, where the sequence is present in double-stranded form, the sequence will be denatured. Denaturation can be carried out by various techniques, such as alkali, generally from about 0.05 to 0.2 M hydroxide, formamide, chaotropic salts, heat, or combinations thereof.

In a first step, the analyte may be immobilized directly upon a solid phase or by sandwich hybridizations in which the analyte is bound to an oligonucleotide that is in turn bound to a solid phase. A particularly useful approach is a solution phase sandwich hybridization described in commonly owned EPA publication No. 0225807.

In a sandwich hybridization assay with a capture step the template probe is used as follows: Single-stranded analyte nucleic acid is incubated under hybridization conditions with an excess of two

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single-stranded nucleic acid probes, (1) an analyte capture probe having a first binding sequence complementary to the analyte and a second binding sequence that is complementary to a single-stranded oligonucleotide bound to a solid phase, and (2) a template linker probe having a first binding sequence that is complementary to the analyte and a second binding sequence that is complementary to domain A of the template probe.

10 In a preferred embodiment, a set of analyte capture probes may be used wherein each member of the set has a different first binding sequence complementary to a different segment of the analyte while all members of the set have the same second binding sequence. Similarly, a  
15 set of template linker probes may be used wherein each member of the set has a different first binding sequence complementary to a different segment of the analyte, but all members of the set have the same second binding sequence complementary to domain A of the template probe.  
20 This approach has the advantage of enabling the simultaneous detection of closely related variants of an analyte, e.g. the genomes of related viral strains.

By using analyte capture and template linker probes, the solid matrix and the template probe can be  
25 used as a "universal" reagent and different immobilized oligonucleotide matrices and template probes need not be made for each analyte.

Usually, hybridization conditions consist of an aqueous medium, particularly a buffered aqueous medium,  
30 which includes various additives. These additives include the polynucleotides to be hybridized, salts (e.g., sodium citrate 0.017M to 0.17M and sodium chloride 0.17M to 1.7M), nonionic or ionic detergents (0.1 to 1.0%), and carrier nucleic acids. Nonaqueous solvents such as  
35 dimethylformamide, dimethylsulfoxide, and formamide may

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also be used. The mixture is incubated for 15 to 75 minutes at 45°C to 70°C. The stringency of the hybridization is regulated by temperature and salt concentration and may be varied depending on the size and homology of the sequences to be hybridized. For hybridization of sequences to bound DNA, the empirical formula for calculating optimum temperature under standard conditions (0.9 M NaCl) is:

$$T(^{\circ}\text{C}) = 4(N_G + N_C) + 2(N_A + N_T) - 5^{\circ}\text{C},$$

where  $N_G$ ,  $N_C$ ,  $N_A$ , and  $N_T$  are the percentage of G, C, A, and T bases in the sequence (Meinkoth, J., et al., Anal. Biochem. (1984) 138:267-284).

The resulting product is a three component nucleic acid complex of the two probes hybridized to the analyte by their first binding sequences. The second binding sequences of the template linker probe and analyte capture probe remain as single-stranded tails as they are not complementary to the analyte.

This complex is then added under hybridizing conditions to a solid phase having a single-stranded oligonucleotide bound to it that is complementary to the second binding sequence of the analyte capture probe. The resulting product comprises the complex bound to the solid phase via the duplex formed by the oligonucleotide bound to the solid phase and the second binding sequence of the analyte capture probe. The solid phase with bound complex is then separated from unbound materials and washed to remove any residual unbound material.

The template probe is then added to the solid phase-analyte-probe complex under hybridization conditions to permit the template probe to hybridize to the available second binding sequences of the template linker probe of the complex (the target sequence of the template probe). The resulting solid phase complex is

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then separated from any unbound template probe and washed.

Next, the RNA polymerase specific for the promoter region (domain B) of the template probe is added, under appropriate transcription conditions and multiple RNA copies (c) of the C domain template (c') are produced. The amount of transcript is proportional to the quantity of the analyte in the initial preparation.

Transcription conditions consist of an aqueous medium, preferably a buffered aqueous medium, with appropriate salts, usually including a magnesium salt, a mixture of NTPs (rATP, rUTP, rGTP, rCTP), a RNA polymerase enzyme and usually include various denaturing agents, protein carriers, and RNase inhibitors. Incubation is usually for 15 to 90 minutes, usually 60 minutes; and at a temperature which is optimal for the chosen enzyme, usually 35°C to 42°C, usually 37°C.

The sequence of the C domain is designed for a specific detection scheme and several such schemes may be employed to quantify the transcripts. For example, the transcription product (c) of the C domain may be subdivided into 2 subdomains-- $c_1$  and  $c_2$  (Figure 2B). Subdomain  $c_1$  is complementary to a transcript capture probe which has been immobilized on a solid substrate. Subdomain  $c_2$  is complementary to an amplifier probe. After hybridization the amount of label retained is linearly proportional to the amount of analyte present in the original sample. In a variation of this approach, the transcripts are sandwiched with linker probes, i.e., the transcript capture probe is in solution rather than immobilized, and contains a second domain which is complementary to an immobilized oligonucleotide; and subdomain  $c_2$  is complementary to an amplifier linker probe which in turn is complementary to the amplifier probe. This sandwiching arrangement is similar to the

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use of analyte capture and template linker probes to sandwich the analyte as described above.

In an alternate embodiment the transcript of the C domain has only a  $c_1$  subdomain. The C domain is transcribed in the presence of labeled ribonucleotide triphosphates and the labeled transcript is subsequently bound to an immobilized transcript capture probe through its complementary  $c_1$  subdomain and quantified.

In yet another embodiment the transcript of the C domain has only a  $c_2$  subdomain. The C domain is transcribed in the presence of biotinylated ribonucleoside triphosphates and the transcripts is captured on avidin beads. The transcript is then annealed to an amplifier probe through its complementary  $c_2$  subdomain and quantified.

Several other methods of labeling and detecting the transcript of the amplifying probe are possible, including the simultaneous use of labeled ribonucleotides and avidin/biotin coupling, and will be obvious to those skilled in the art.

#### Capture, Linker and Amplifier Probes

The first binding sequences of the analyte capture probe and template linker probe are complementary to the analyte sequence. Similarly, the first binding sequences of the transcript capture and amplifier linker probes are complementary to the reporter transcripts. Each first binding sequence is at least 12 nucleotides (nt), usually at least 25 nt, more usually at least 30 nt, and not more than about 150, usually not more than about 75, preferably not more than about 50 nt. They will normally be chosen to bind to different sequences of the analyte. The first binding sequences may be selected based on a variety of considerations. Depending upon the nature of the analyte, one may be

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interested in a consensus sequence, a sequence associated with polymorphisms, a particular phenotype or genotype, a particular strain, or the like.

5 The second binding sequences of the analyte capture probe and template linker probe are selected to be complementary, respectively, to the oligonucleotide attached to the solid phase and to an oligonucleotide unit of the template probe and so as to not be encountered by endogenous sequences in the sample/analyte.

10 The second binding sequence may be contiguous to the first binding sequence or be spaced therefrom by an intermediate noncomplementary sequence. The probes may include other noncomplementary sequences if desired. These noncomplementary sequences must not hinder the

15 binding of the binding sequences or cause nonspecific binding to occur.

The capture probes and linker probes may be prepared by conventional oligonucleotide synthesis procedures or by cloning.

20 It will be appreciated that the binding sequences need not have perfect complementarity to provide homoduplexes. In many situations, heteroduplexes will suffice where fewer than about 10% of the bases are mismatches, ignoring loops of five or more numbers.

25 Accordingly, as used herein the term "complementary" intends a degree of complementarity sufficient to provide a stable and specific duplex structure.

The solid phase that is used in the assay may be particulate or be the solid wall surface of any of a variety of containers, e.g., centrifugal tubes, columns,

30 microtiter plate wells, filters, tubing, etc. Preferably, particles will be employed of a size in the range of about 0.4 to 200 microns, more usually from about 0.8 to 4.0 microns. The particles may be any

35 convenient material, such as latex, or glass. The

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ligonucleotide that is complementary to the second binding sequence of the analyte capture probe may be stably attached to the solid surface through functional groups by known procedures.

5 It will be appreciated that one can replace the second binding sequence of the capture probe and the oligonucleotide attached to the solid phase with an appropriate ligand-receptor pair that will form a stable bond joining the solid phase to the first binding  
10 sequence of the capture probe. Examples of such pairs are biotin/avidin, thyroxine/thyroxine-binding globulin, antigen/antibody, carbohydrate/lectin, and the like.

The amplifier probes will include a sequence complementary to the C<sub>2</sub> subdomain of the transcripts of  
15 the template probe, or to a subdomain of an amplifier linker probe. The amplifier probe is capable of hybridizing to one or more labels or labeling probes which directly or indirectly provide for a detectable signal. The labels may be incorporated in individual  
20 residues of the complementary sequence or may be present as a terminal domain or terminal tail having a plurality of labels. Various means for providing labels bound to the sequence have been reported in the literature. See, for example, Urdea et al., Nucl. Acids Res. (1988) 4937;  
25 Leary et al., Proc. Natl. Acad. Sci. USA (1983) 80:4045; Renz and Kurz, Nucl. Acids Res. (1984) 12:3435; Richardson and Gumpert, Nucl. Acids Res. (1983) 11:6167; Smith et al., Nucl. Acids Res. (1985) 13:2399; Meinkoth and Wahl, Anal. Biochem. (1984) 138:267. The labels may  
30 be bound either covalently or noncovalently to the complementary sequence. Labels which may be employed include radionuclides, fluorescers, chemilumescers, dyes, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, enzyme subunits, metal ions, and the  
35 like. Illustrative specific labels include fluorescein,

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rhodamine, Texas red, phycoerythrin, umbelliferone, luminol, NADPH, galactosidase, horseradish peroxidase, alkaline phosphatase, etc. See Urdea et al. for a comparison of nonradioisotopic labeling methods.

5           The labeling probes can be conveniently prepared by chemical synthesis such as that described in commonly owned copending application Serial No. 945,876. By providing for a terminal group which has a convenient functionality, various labels may be joined through the  
10           functionality. Thus, one can provide for a carboxy, thiol, amine, hydrazine or other functionality to which the various labels may be joined without detrimentally affecting duplex formation with the sequence. As already indicated, one can have a molecule with a plurality of  
15           labels joined to the sequence complementary to the labeling sequence. Alternatively, one may have a ligand bound to the labeling sequence and use a labeled receptor for binding to the ligand to provide the labeled analyte complex.

20           The ratio of analyte capture probe and template linker probe to anticipated moles of analyte will each be at least stoichiometric and preferably in excess. This ratio is preferably at least about 1.5:1, and more preferably at least 2:1. It will normally be in the  
25           range of 2:1 to 10,000:1. Concentrations of each of the probes will generally range from about  $10^{-9}$  to  $10^{-6}$  M, with sample nucleic acid concentrations varying from  $10^{-21}$  to  $10^{-12}$  M. The hybridization steps of the assay  
30           will generally take from about 10 minutes to 2 hours, frequently being completed in about 1 hour. Hybridization can be carried out at a mildly elevated temperature, generally in the range from about 20°C to 80°C, more usually from about 35°C to 70°C, particularly 65°C. Additional conditions for the hybridization  
35           reaction are described infra.



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The procedure used in the separation steps of the assay will vary depending upon the nature of the solid phase. For particles, centrifugation or filtration will provide for separation of the particles, discarding  
5 the supernatant or isolating the supernatant. Where the particles are assayed, the particles will be washed thoroughly, usually from one to five times, with an appropriate buffered medium containing detergent, e.g., PBS with SDS. When the separation means is a wall or  
10 support, the supernatant may be isolated or discarded and the wall washed in the same manner as indicated for the particles.

Depending upon the nature of the label, various techniques can be employed for detecting the presence of  
15 the label. For fluorescers, a large number of different fluorometers are available. With enzymes, either a chemiluminescent, fluorescent or a colored product can be provided and determined fluorometrically, spectrophotometrically or visually. The various labels  
20 which have been employed in immunoassays and the techniques applicable to immunoassays can be employed with the subject assays.

In a hybridization assay in which the analyte nucleic acid is bound directly to a solid phase, such as  
25 a "dot blot" assay, the template probe is hybridized directly to the bound analyte. In these instances, the A domain of the template probe is complementary to a sequence of the analyte.

The template probe may also be used in other  
30 assays such as direct, indirect, and sandwich immunoassays and assays for ligand receptors, for instance cell surface receptors. In these instances, rather than a label, the reagent that plays the role of the labeled antibody, or other ligand which binds to the analyte  
35 (antigen or ligand receptor), has an attached oligonu-

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cleotide that is complementary to a', the sequence of the A domain of the template probe. For instance, in a sandwich immunoassay for an antigen analyte, the analyte sample is incubated with a solid phase to which is bound a first antibody to the antigen. Unbound sample is removed from the solid phase and a second antibody to the antigen, which is coupled to an oligonucleotide complementary to a', is reacted with the bound complex to form a three-membered complex. Following removal of excess second antibody the template probe is then hybridized to the complex via the oligonucleotide bound to the second antibody. Excess template probe is removed and RNA polymerase is added as described supra. Finally, the transcription product is quantified as described.

In an alternative embodiment, the template probe may be synthesized without an A domain and coupled directly to the ligand receptor or antibody by means of avidin/biotin or an equivalent stably bonding pair as described previously. The template probe may also be covalently attached to the ligand receptor by means of the chemical synthesis described in copending application No. 945,876.

Kits for carrying out amplified nucleic acid hybridization assays according to the invention will comprise in packaged combination the following reagents: the template probe; the appropriate DNA-directed RNA polymerase; an appropriate labeling probe; a solid phase that is capable of binding to the analyte; optionally an analyte capture probe if the assay format is one in which the analyte is bound to the solid phase through an intermediate oligonucleotide or other ligand; and optionally a template linker probe if the assay format is one in which the template probe is not hybridized directly to the analyte. Similarly, these kits may also contain transcript capture probes amplifier probes and amplifier

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linker probes. These reagents will typically be in separate containers in the kit. The kit may also include a denaturation reagent for denaturing the analyte, hybridization buffers, wash solutions, negative and positive controls and written instructions for carrying out the assay.

### EXAMPLES

10

#### Example 1

##### A. T7 Template Probe.

The probe was designed as shown in Figure 1A. The a' sequence of the A domain is shown in Figure 1B and is complementary to the a region of the template linker probe depicted in Figure 2A. The sequence of the promoter domain, B, (shown in Fig. 1B) contained the consensus T7 promoter sequence plus 15 additional bases 5' to the promoter and identical to the sequence of the pT7 plasmid (available from US Biochemicals) up to the PvuII restriction site. The additional 15 basepairs may be extraneous; however, they were incorporated since the initial experiments conducted with template probes that had been cloned into the pT7 vector proved successful. Thus, even template probes made by chemical synthesis have retained this plasmid portion. The C domain was designed as a random sequence. It was evaluated by computer analysis to minimize potential hybridization cross-reactivity with other probes in the system.

30

##### B. T3 Template Probe.

The probe is designed as in Example 1A, above, except that the consensus sequence for the DNA-directed RNA polymerase promoter sequence of bacteriophage T3 (5'-TATTAACCCTCACTAAA-3') is substituted for the consensus sequence for the T7 promoter (5'-TAATACGACTCACTATA-3').

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C. SP6 Template Probe.

The probe is designed as in Example 1A, above, except that the consensus sequence for the DNA-directed RNA polymerase promoter sequence of bacteriophage SP6 (5'-ATTTAGGTGACACTATA-3') is substituted for the consensus sequence for the T7 promoter and the first six 5' nucleotides of domain C are 5'-GAAGGG-3' rather than 5'-GGGAGA-3, as is the case in Example 1A.

Example 2Hybridization Assay for the Pilin Gene DNA of Neisseria gonorrhoeae Using a Microtiter Dish Assay Procedure and the T7 RNA polymerase.A. Standard Analyte DNA.

The N. gonorrhoeae strain 31707 from the Neisseria Reference Laboratory (Seattle, WA) was used. DNA was prepared from this strain, as well as from several nonpathogenic commensal strains of Neisseria used as controls, by the addition of a proteinase K/SDS solution as described in Urdea et al. (Gene (1987) 61:253).

B. Oligonucleotide Bound to Solid Support (Figure 2A).

A microtiter dish assay procedure was employed. Microtiter dishes were prepared as follows. Two types of microtiter dish wells were prepared: (1) N wells for sample work-up and negative controls, and (2) S wells for capture of the probe-analyte complex from samples and positive controls.

N wells were produced as follows: 300 µl of HM buffer (0.1% SDS, 4xSSC, 1 mg/ml sonicated salmon sperm DNA, 1 mg/ml poly A, 10 mg/ml BSA) was added to Immulon

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II Remov-a-wells (Dynatech Inc.). The well strips were covered and left standing at room temperature for 1 hour.

The HM buffer was removed by aspiration and the wells were washed 3 times with 400  $\mu$ l of 1 x PBS. The strips  
5 were covered with plastic wrap and stored at 4°C until used.

S wells were prepared from the Immulon II strips as follows. To each well, 200  $\mu$ l of a 200  $\mu$ g/ml solution of poly-phenylalanyl-lysine (Sigma Chemical  
10 Inc.) in water. The covered strips were left at room temperature for 30 min to 2 hr, then washed as above.

Next, a 21 base oligomer,  
5'-XCACCACTTCTCCAAAGAAG-3', where X represents the  
N4-(6-aminocaproyl-2-aminoethyl) derivative of 5-methyl  
15 cytidine, was synthesized according to the method of Warner et al. (DNA (1984) 3:401) and purified as described by Urdea et al., supra. The N4-modified cytosine base facilitates the chemical cross-linking of the oligonucleotide as described in commonly owned EPA  
20 Publication No. 0225807 and Urdea, M.S., et al., Nucl. Acids Res. (1988) 16:4937-4956.

A 10 OD sample of the synthesized oligonucleotide in 60  $\mu$ l of 1 x PBS was treated with 140  $\mu$ l of dimethylformamide containing 10 mg of ethylene glycol bis  
25 (succinimidylsuccinate) (Pierce Chemical Inc.). The mixture was vortexed and incubated in the dark at room temperature. After 15 min, the solution was passed over a Sephadex® G-25 column (PD-10 from Pharmacia), previously equilibrated with 30 ml of 1 x PBS. The void  
30 volume of the column was diluted to a final volume of 35 ml with 1 x PBS. To each well, a 50  $\mu$ l aliquot of the oligonucleotide solution was added. After covering with plastic wrap, the wells were incubated at room temperature in the dark for 30 min to overnight. The  
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wells were washed with 1 x PBS, then coated with HM buffer, washed, and stored as above.

C. Analyte Capture Probes (Figure 2A).

5 A set of 3 single-stranded oligomers each having a varying 30 base long portion complementary to a specific sequence of the pilin gene and a constant 20 base long 5'-portion complementary to the oligonucleotide bound to the solid phase was synthesized by the automated  
10 phosphoramidite procedures described in Warner et al., supra, and purified by the method of Sanchez-Pescador and Urdea, supra. The sequences complementary to the pilin gene were based on the N. gonorrhoeae pilin sequence described by Bergstrom, S., et al. (PNAS USA (1986)  
15 83:3890-3894). The 5' portions of the probes were complementary to segments of the pilin sequence and were as follows:

	<u>Probe Designation</u>	<u>5'-Sequence</u>
20	GCP-XT1-4	GAT GTG GCG GGC GCG CGT TCA AAG GCT TCG
	GCP-XT1-8	GAG GCT GTA GTT TCC GTT TAT ACA ATT TCT
	GCP-XT1-12	GCC AAG CCA TTT TAC CAA GAC GCC TGT CGG

25 The 3'-portion of each analyte capture probe was constructed to be complementary to the sequence of the oligonucleotide attached to the solid support described infra.

D. Template Linker Probes (Figure 2A).

30 A set of 12 single-stranded oligomers each consisting of a varying 30 base long portion complementary to a specific sequence of the pilin gene and a constant : base long 3'-portion complementary to the template probe (Figure 2A) were synthesized by the  
35

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procedures of Warner et al., supra and purified according to Sanchez-Pescador and Urdea, supra.

The 5' portions of the probes were complementary to segments of the pilin sequence and were as follows:

	<u>Probe Designation</u>	<u>5'-Sequence</u>
	GCP-LLA2C-1	ATA CTT ATG GGA AGT TTT TCC GAA ATG GGA
10	GCP-LLA2C-2	GCT CGA CTA CTA ACA CTA GCG ATA GCA GCC
	GCP-LLA2C-3	AAA CCG CAA TCA GCG GGA AGG GCG GAT GGT
	GCP-LLA2C-5	GGA AAA CCG GCT TCC AGT TTT TAG TCG GCA
	GCP-LLA2C-6	GCT CAT AAT GGA CTT AAG GCC GTT TAC CGG
	GCP-LLA2C-7	TTT GTT GTG AAG ACG GCC GCA CCG TAG GGG
15	GCP-LLA2C-9	ACT TCA ATT TTT GCC GCA GCA ATG GCG GTG
	GCP-LLA2C-10	CGA AAG TTC GCC GCA TTT GTT ACT AAT GTT
	GCP-LLA2C-11	GTT TTT TGA GAG GGA CAC CCG GTC CGC ACT
	GCP-LLA2C-13	ATG CGC GTG GCT GCT GCT GTG GCA ACG GCT
	GCP-LLA2C-14	GTT TCT GCC GTT TCT TTA GCT GTG GTT CGT
20	GCP-LLA2C-15	CGG CAG TTG GAC GGC GCT ATT CCG TAG ACT

The 3'-portion of each template linker probe was constructed to be complementary to the sequence of the A domain of the template probe.

25

#### E. Labeled Oligomer (Figure 2B).

An 18 base oligomer, 5'-XGGTCCTAGCCTGACAGC-3', where X is defined as above, was synthesized as described, and combined with alkaline phosphatase (AP) as follows: Calf intestinal AP (3mg in buffer; immunoassay grade, Boehringer-Mannheim) was placed in a Centricon 30 Microconcentrator. Approximately 2 ml of 0.1 M sodium borate, pH 9.5, was then added and the device was spun at 3500 rpm until a final volume of 40  $\mu$ l was obtained. The alkylamino oligonucleotide was then activated with p-

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phenylene diisothiocyanate (DITC; Pierce Chemicals) in 95:5 (v/v) dimethylformamide: 0.1 M sodium borate, pH 9.3, extracted with *n*-butanol, and combined with the protein. The final product was stored at 4°C. See Urdea  
5 et al. (Nuc. Acids Res. (1988) 16:4937).

#### F. Microtiter Dish Procedure.

For duplicate analyses, 20 µl of each sample was placed into 2 N wells, then treated with 25 µl of  
10 proteinase K/SDS solution. The wells were covered with a Linbro-Titertek microtiter plate sealer, gently agitated, and incubated at 65°C for 30 min in a water bath. The analyte capture and template linker probe sets in a 1 M NaOH were added in 10 µl to each well. After sealing,  
15 the samples were incubated for 10-30 min at 65°C to 72°C as above. The solutions were neutralized with 26 µl 0.38 M acetic acid (or 0.76 M 3-[N-morpholino] propane sulfonic acid (MOPS), free acid), 12.3 x SSC, then incubated for an additional 15-30 min covered at 65°C.  
20 From each N well, 40 µl of sample was transferred to a new S well containing the solid supported capture probe. The wells were sealed and set at 65°C for 1 hour. Each well was then washed 2 times by aspiration with 0.1% SDS, 0.1 x SSC. See Folberg et al. (Molec. and Cell. Probes  
25 (1989) 3:59).

The template probe was subsequently annealed to the complex by incubation of 100 fmoles of template probe in 40 µl of 4 x SSC with 100 µg/ml poly A at 55°C for 1 hr followed by two washes with 0.1 x SSC, 0.1% SDS and  
30 two washes with 0.1 x SSC.

Transcription of domain C was effected by incubating the complex in 20 µl of a solution containing 40 mM Tris HCl (pH 8), 20 mM MgCl<sub>2</sub>, 10 mM NaCl, 1 mM Spermidine, 10 mM Dithiothreitol, 0.15 mg/ml Bovine Serum  
35 Albumin, 1.25 mM each of rATP, rCTP, rGTP, rUTP, 1600



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units/ml RNasin, and 2000 units/ml T7 RNA polymerase. This mixture was incubated at 37°C for 1 hour. Transcription was terminated by addition of 20 µl of a solution containing 8 X SSC, and 0.2% SDS and the entire  
5 mixture was transferred to new wells containing an immobilized capture probe with c<sub>1</sub>' sequences. Capture of the domain C transcripts (Figure 2B) was effected by incubation at 55°C for 1 hour followed by two washes with 0.1 X SSC, 0.1% SDS.

10 The domain C transcripts were then labeled by addition of 50 fmol of enzyme-labeled probe (c<sub>2</sub>') in 40 µl of 4 X SSC, 100 µg/ml poly A for 15 min. at 55°C. Finally, the complex was washed twice with 0.1 X SSC, 0.1% SDS, followed by two washes with 0.1X SSC.

15 For AP detection, an enzyme-triggered dioxetane-based reaction (Schapp et al. Tet. Lett. (1987) 28:1159-1162) and US Patent 4,857,652), available from Lumigen Inc., was employed. The detection procedure was as follows. For the labeling step 40 µl HM buffer with  
20 the AP probe was added to each well and the wells were incubated at 55°C for 15 min. The supernatant was removed and the wells were washed 2 x with 380 µl of 0.1 x SSC and 0.1% SDS. The wells were then washed 2 x with 380 µl of 0.1 x SSC to remove any remaining SDS. 20 µl  
25 of 3.3 x 10<sup>-4</sup> M dioxetane reagent in CTAB buffer was added to each well. The wells were tapped lightly so that the reagent would fall to the bottom and gently swirled to distribute the reagent evenly over the bottom. The wells were covered with the microtiter plate sealer  
30 and incubated in a 37°C oven for one hour. The wells were then read with a luminometer.

### Results

Tests were carried out on N. gonorrhoeae  
35 bacterial cells (strain 31707) as well as nonpathogenic

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Neisseria controls according to the protocol of Example 2, above. Results are presented as a signal to noise ratio (S/N) representing the value of the sample versus the value of the control. Cell number was  
 5 determined by cell viability. For comparison, tests were also carried out on the same samples using a branched 5-site comb-type amplification multimer described in copending application U.S. Serial No. 109,282.

Cell Number	T7 Transcription Assay		Multimeric Assay	
	Trial 1	Trial 2	Trial 1	Trial 2
8.3 x 10 <sup>5</sup>	186.76±25.39	139.93±44.87	90.94±48.69	82.75±16.55
15 8.3 x 10 <sup>6</sup>	18.35± 3.60	8.35± 4.20	9.94± 8.71	14.56± 0.47
8.3 x 10 <sup>3</sup>	2.43± 0.37	1.55± 0.37	2.58± 1.40	2.55± 0.42

20

Example 3Hybridization Assay Using the T3 RNA Polymerase

A hybridization assay is employed using the same protocol as in Example 2, supra, except that domain B of the template probe contains the sequences for the T3  
 25 RNA polymerase promoter rather than the T7 promoter. The sequence of the T3 promoter has been previously disclosed by Brown, J.E., et al. (Nucleic Acids Res. (1986) 14:3521-3526). The T3 promoter sequence is 5'-TAT TAA CCC  
 TCA CTA AAG GGA GA-3' and replaces the T7 promoter  
 30 sequence 5'-TAA TAC GAC TCA CTA TAG GGA GA-3'.

Example 4Hybridization Assay Using the SP6 RNA Polymerase

A hybridization assay is employed using the  
 35 same protocol as in Example 2, supra, except that domain

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B of the template probe contains the sequence for the SP6 RNA polymerase promoter rather than the T7 promoter. The sequence of the SP6 promoter has been previously disclosed by Brown et al., *supra*. The SP6 promoter sequence is 5'-ATT TAG GTG ACA CTA TAG AAG GG-3' and replaces the T7 promoter sequence 5'-TAA TAC GAC TCA CTA TAG GGA GA-3'.

#### Example 5

#### 10 Hybridization Assay for Hepatitis B Virus (HBV) DNA Using the Microtiter Dish Assay Procedure and T7 RNA Polymerase

DNA extracts of serum or plasma samples of patients potentially infected with hepatitis B virus (HBV) are prepared as described in copending US application No. 109,282 and are used as analyte as described in Example 2.

A set of single-stranded template linker probes, each having a varying 30 base long portion complementary to a specific sequence of the constant ds region of the HBV genome and a constant 20 base long 3'-portion complementary to the template probe used in Example 2 is synthesized by the procedures described in Example 2. The sequences of these probes are presented in Table 1 below.

TABLE 1

#### Template Linker Probes for HBV

<u>Probe Designation</u>	<u>Sequence</u>
30 :HBV.LLA2C.70	TGA CTG [CG]CG ATT GGT [GA]GA GGC AGG [AC]GG AGG TTA GGC ATA GGA CCC GTG TC
:HBV.LLA2C.69	CTT G[AT][CT] GGG [GA]TT GAA GTC CCA ATC TGG ATT TTA GGC ATA GGA CCC GTG 35 TC

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:HBV.LLA2C.68 GTT GCG TCA GCA AAC ACT TGG CA[CG]  
AGA CC[AT] TTA GGC ATA GGA CCC GTG TC

5 :HBV.LLA2C.67 TAA GTT GGC GAG AAA GT[GA] AAA GCC  
TG[TC] TT[AC] TTA GGC ATA GGA CCC GTG  
TC

10 :HBV.LLA2C.66 GCA GCA AA[GA] CCC AAA AGA CCC ACA  
A[TG][TA] C[TG][TC] TTA GGC ATA GGA  
CCC GTG TC

15 :HBV.LLA2C.65 ATG TAT ACC CA[GA] AGA CA[AG] AAG AAA  
ATT GGT TTA GGC ATA GGA CCC GTG TC

:HBV.LLA2C.59 TAG AGG ACA AAC GGG CAA CAT ACC TTG  
[AG]TA TTA GGC ATA GGA CCC GTG TC

20 :HBV.LLA2C.58 GAT GAG GCA TAG CAG CAG GAT GAA GAG  
GAA TTA GGC ATA GGA CCC GTG TC

:HBV.LLA2C.57 GAT AAA ACG CCG CAG ACA CAT CCA GCG  
ATA TTA GGC ATA GGA CCC GTG TC

25 :HBV.LLA2C.56 GGA CAA [AG]TT GGA GGA CA[GA] GAG GTT  
GGT GAG TTA GGC ATA GGA CCC GTG TC

:HBV.LLA2C.55 TTG GAG GTT GGG GAC TGC GAA TTT TGG  
CCA TTA GGC ATA GGA CCC GTG TC

30 :HBV.LLA2C.54 CCA CCA CGA GTC TAG ACT CTG [CT]GG  
TAT TGT TTA GGC ATA GGA CCC GTG TC

:HBV.LLA2C.53 GAT TCT TGT CAA CAA GAA AAA CCC CGC  
CTG TTA GGC ATA GGA CCC GTG TC

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:HBV.LLA2C.52      CAC GAG [CA]AG GGG TCC TAG GAA TCC  
                          TGA TGT TTA GGC ATA GGA CCC GTG TC

5    :HBV.LLA2C.51      CAG GGT TTA CTG TTC C[TG]G AAC TGG  
                          AGC CAC TTA GGC ATA GGA CCC GTG TC

A set of single-stranded analyte capture probes, each having a varying 30 base-long portion complementary to a specific sequence of the constant ds region of the HBV genome and a constant 20 base long 3'-portion complementary to the oligonucleotide bound to a microtiter dish as described in Example 2 is synthesized as described in Example 2. The sequences of these probes are presented in Table 2 below.

**TABLE 2**  
**Analyte Capture Probes for HBV**

	<u>Probe Designation</u>	<u>Sequence</u>
20	:HBV.XT1.64	CTT GGC CCC CAA TAC CAC ATC ATC CAT ATA CTT CTT TGG AGA AAG TGG TG
	:HBV.XT1.63	GAA AGC CAA ACA GTG GGG GAA AGC CCT ACG CTT CTT TGG AGA AAG TGG TG
25	:HBV.XT1.62	CAC TGA ACA AAT GGC ACT AGT AAA CTG AGC CTT CTT TGG AGA AAG TGG TG
30	:HBV.XT1.61	GAG AAA CGG [AG]CT GAG GCC C[AC]C TCC CAT AGG CTT CTT TGG AGA AAG TGG TG
	:HBV.XT1.60	[GC]CG AAA GCC CAG GA[CT] GAT GGG ATG GGA ATA CTT CTT TGG AGA AAG TGG TG

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All other methods and reagents are the same as Example 2.

Example 6

Hybridization Assay for TEM-1 beta-Lactamase DNA in *N. gonorrhoeae* Using the Microtiter Dish Assay Procedure and T7 RNA polymerase

5 Molecular analyses have revealed that the penicillin resistance observed in *N. gonorrhoeae* is  
10 mostly due to the presence of a TEM-1 beta-Lactamase gene in a nonconjugative plasmid of 3-7 M. daltons. (This plasmid is homologous to those found in *H. ducreyi*, *H. parainfluenzae*, and occasionally *H. influenzae*.) A hybridization assay is thus developed to detect TEM-1 DNA  
15 in *N. gonorrhoeae* (or the other aforementioned bacteria carrying homologous plasmids) for the purpose of determining penicillin resistance.

The 7.3 Kb *N. gonorrhoeae* plasmid carrying the TEM-1 gene has been obtained and a segment containing 80%  
20 of the TEM-1 gene was sequenced as described in commonly owned EPA Publication No. 0317077. Analyte capture and template linker probes are synthesized and purified as described in Example 2, supra. The 5'-portion of the template linker probes are complementary to sequences of  
25 the coding region of the gene; whereas the 5'-portions of the analyte capture probes are complementary to adjoining sequences of the plasmid. Alternatively, probes are also prepared in which the 5'-portions of both sets are directed to the TEM-1 gene.

30 In all other respects the hybridization assay procedure and reagents are the same as described in Example 2.

This TEM-1 assay is a powerful clinical tool that will enable medical personnel to identify  
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penicillin-resistant infection and optimize a treatment regime by choosing an appropriate antibiotic therapy.

#### Example 7

##### 5    Hybridization Assay for Chlamydia trachomatis DNA Using the Microtiter Dish Assay Procedure and T7 Polymerase

Template linker and analyte capture probes are prepared using the same strategy as described in Example 2 and designed to hybridize to the Chlamydia  
10    pCHL2 plasmid described by Palmer and Falkow (Plasmid (1986) 16:52-62). Each probe of the set is a 50 mer in which the first 30 5'-residues are complementary to pCHL2 sequences and the remaining 3'-residues are the system-specific analyte capture and template linker sequences  
15    described in Example 2. The pCHL2 sequences used to design these probes are disclosed in commonly owned EPA Publication No. 0317077.

In all other respects the hybridization assay procedure and reagents are the same as describe in  
20    Example 2.

#### Example 8

##### Hybridization Assay for tet M Determinant in N. gonorrhoeae

25    N. gonorrhoeae strains resistant to high levels of tetracycline, exhibiting minimum inhibitory concentration values above 16 g/ml, have been found to have acquired the tet M determinant in a 24.5 Md conjugative plasmid (Cannon, J.G., et al., Annual Review of  
30    Microbiology (1984) 38:111; Morse, S.A., et al., Antimicrob. Agents Chemother. (1986) 30:664). A hybridization assay is thus developed to detect tet M DNA in N. gonorrhoeae (or the other aforementioned bacteria carrying homologous plasmids) for the purpose of  
35    determining tetracycline resistance.

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Ten  $\mu$ l of tetracycline resistant N. gonorrhoeae (TRNG) cells suspended in either GC broth or skimmed milk are mixed with 12.5  $\mu$ l of lysis solution (2mg/ml proteinase K in 10mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 1% SDS, pH 8.0) in a clear Immulon II well (Dynatech), and incubated at 65°C for 20 min.

Analyte capture and template linker probes are synthesized and purified as described in Example 2, supra, except they are designed to hybridize to the tet M structural gene. The sequences of the probes are based on the tet M gene sequence from the streptococcal conjugative shuttle transposon Tn 1545 described in Martin, P., et al., Nuc. Acids Res. (1986) 14:7047.

In all other respects the hybridization assay procedure and reagents are the same as described in Example 2.

#### Example 9

Comparison of Template Probes with Various Numbers of Base Pairs Between the A and B Domain in Assays for the Presence of Human Immunodeficiency Virus (HIV) DNA in Human Plasma.

Various template probes were prepared, each having the same functional domains, A, B, and C, but with different numbers of base pairs separating the A domain and the T7 promoter.

The general strategy was to prepare DNA containing the T7 polymerase promoter operably linked to the 5' end of the Hepatitis B viral genome (HBV) - about 3.4 Kb in length. Thus, the HBV genome acts as domain C and functions as the template for subsequent transcription while the resulting HBV-specific RNA functions as reporter transcripts. This DNA fragment was cloned in plasmid pGEM3Z (commercially available from



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Promega, Inc.- see Figure 3A). Next, a partially single stranded oligomer, corresponding to the A domain of the template probes, and a short (18 nucleotide) double stranded spacer region with a cohesive end was also prepared (Figure 4). The oligomer was then ligated to the promoter/HBV DNA fragment (B/C domain) which had been isolated from the plasmid by restriction endonuclease digestion and subsequent purification. The size of the fragment varied depending on the restriction endonuclease used to linearize the plasmid (Figures 3A-C).

#### A. pII template probe

##### 1. T7 promoter (domain B) and HBV (domain C)

A DNA segment comprised of the T7 consensus sequence, oriented directly 5' to linearized HBV genomic DNA, was inserted in the EcoRI site of pGEM3Z (pGEM3Z-HBV). After cloning, the plasmid was isolated and relinearized by digestion with Nde I (Figure 3A).

##### 2. The Oligomer

A partially double stranded oligomer was synthesized which comprised domain A and a short double stranded domain terminating in a cohesive end complementary to the cohesive end created by Nde I digestion. This oligomer is designated oligo N. Oligo N is comprised of two DNA strands. The sequence of strand X is 5'-GGT CGA CTA ATC GGT AGC-3'. The sequence of strand Y is 3'-TCC GTA TCC TGG GCA CAG CCA GCT GAT TAG CCA TCG AT-5'. Strands X and Y were annealed creating a single stranded domain A at the 3'-end of strand Y and an AT cohesive end at the 5'-end of strand Y.

##### 3. Ligation

Oligo N and Nde I linearized pGEM3Z-HBV were ligated, creating a oligonucleotide with oligo N at both ends of the molecule (Figure 3A). The molecule was trimmed with Hind III thereby creating the template probe

-40-

designated pII (Figure 3A). The distance between the T7 promoter and domain A is 200 base pairs.

B. pII<sub>L</sub> template probe

5 pII<sub>L</sub> template probe was synthesized as described in A, supra, except that pGEM3Z-HBV was linearized with Sca I, thereby creating a longer spacer region between the T7 promoter of domain B and domain A (Figure 3B). In this case, the oligomer is designated  
10 oligo S and consists of strands X and Y'. Oligo S differs from oligo N in that there is no cohesive end. Strand X is the same as strand X in oligo N, but strand Y' lacks the 5'-TA. Strands X and Y' were annealed creating a single stranded domain A at the 3'-end of  
15 strand Y' and a blunt end at the 5'-end of strand Y. The linearized plasmid and oligo S were blunt-end ligated. The distance between the T7 promoter and domain A is 900 base pairs.

20 C. pII<sub>R</sub> template probe

pII<sub>R</sub> template probe was synthesized as described in A, supra, except that pGEM3Z-HBV was linearized with Hind III (Figure 3C). The oligomer is designated oligo H and consists of strands X and Y'.  
25 Oligo H differs from oligo N in that the cohesive end is complementary to the cohesive end generated by Hind III digestion. Strand X is the same as strand X in oligo N, but strand Y'' has the sequence 3'-TCC GTA TCC TGG GCA CAG CCA GCT GAT TAG CCA TCG TCGA-5'. Strands X and Y''  
30 were annealed creating a single stranded domain A at the 3'-end of strand Y'' and an TCGA cohesive end at the 5'-end of strand Y''. Oligo H and Hind III-linearized pGEM3Z-HBV were ligated, creating a oligonucleotide with oligo H at both ends of the molecule (Figure 3C). The  
35 molecule was trimmed with Nde I rather than Hind III,

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thereby creating the template probe designated pII<sub>R</sub>. This probe differs from pII and pII<sub>L</sub> in that the sequence of domains is B-C-A. The distance between T7 promoter and domain A is 3200 base pairs.

5

#### D. HIV-specific capture and linker probes.

The assays described below (see Figure 5) are similar to the assay described in Example 2 above. Since HIV is the analyte it was necessary to create analyte capture probes and template linker probes with subdomains homologous to the HIV genome. The sequences of these novel linker probes are provided below. The 5'-portion of each probe is complementary to a portion of the HIV genome while the 3'-portion is complementary to an immobilized oligonucleotide capture sequence (in the case of capture probes) or to the A domain of the T7 template probe (in the case of the template linker probes).

10  
15  
20

TABLE 3

#### Analyte Capture Probes

<u>Probe Designation</u>	<u>Sequence</u>
:HIV.96.1.XT1	TTC TAC TAC TTT [TC]AC CCA TGC [AG]TT TAA AGC TTC TTT GGA GAA AGT GGTG
:HIV.96.2.XT1	TTC TAT TAC TTT [TC]AC CCA TGC [AG]TT CAA AGC TTC TTT GGA GAA AGT GGT G
:HIV.97.XT1	TGC TTG ATG TCC CCC CAC TGT GTT TAG CAT CTT CTT TGG AGA AAG TGG TG
:HIV.97.2.XT1	TGC CTG GTG TCC TCC AAC TAT GTT CAG CAT CTT CTT TGG AGA AAG TGG TG

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30  
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	:HIV.53.XT1	AGG TGA TAT GGC [CT]TG ATG TA[CT] CAT TTG CCC CTT CTT TGG AGA AAG TGG TG
5	:HIV.54.XT1	CAT GGG TAT [TC]AC TTC TGG GCT [GA]AA [AG]GC CTT CTT CTT TGG AGA AAG TGG TG
10	:HIV.55.XT1	TTG [TC]GG GGT GGC [TC]CC [TC]TC TGA TAA TGC TGA CTT CTT TGG AGA AAG TGG TG
	:HIV.68.1.XT1	AAT TTT T[GA]A AAT TTT [TC]CC TTC CTT TTC CAT CTT CTT TGG AGA AAG TGG TG
15	:HIV.68.2.XT1	AAC TCT T[GA]A AAT TTT [TC]CC TTC CTT TTC CAT CTT CTT TGG AGA AAG TGG TG
	:HIV.99.XT1	TTA CTG GTA CAG T[TC]T CAA TAG G[AG]C TAA T[GT]G CTT CTT TGG AGA AAG TGG TG
20	:HIV.100.XT1	TAA C[TC][TC] TTG GGC CAT CCA T[TC]C CTG GCT TTC TTC TTT GGA GAA AGT GGT G
25	:HIV.101.XT1	CTT TTA TTT TTT CTT CTG TCA ATG GCC ATC TTC TTT GGA GAA AGT GGT G
	:HIV.102.XT1	AAA TAC TGG AGT ATT GTA TGG ATT [CT]TC AGC TTC TTT GGA GAA AGT GGT G

TABLE 4Template Linker ProbesProbe DesignationSequence

:HIV.51.LLA2C

TCC [CG]CC GCT TAA TAC [TC]GA CGC TCT  
CGC ACC TTA GGC ATA GGA CCC GTG TC

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:HIV.52.LLA2C TTA [TA]AT AAT GAT [CT]TA AGT TCT TCT  
GAT CCT TTA GGC ATA GGA CCC GTG TC

5 :HIV.56.LLA2C ACT TCC [CT]CT TGG TTC TCT CAT [CT]TG  
[GA]CC TGG TTA GGC ATA GGA CCC GT GTC

:HIV.57.LLA2C TTC [CT]TG AAG GGT ACT AGT [AG]GT TCC  
TGC TAT TTA GGC ATA GGA CCC GTG TC

10 :HIV.58.1.LLA2C GAT AGG TGG ATT A[CT][TG] TGT CAT CCA  
T[GC]C TAT TTA GGC ATA GGA CCC GTG TC

:HIV.58.2.LLA2C GAT AGG TGG GTT G[TC][TG] TGT CAT CCA  
T[GC]C TAT TTA GGC ATA GGA CCC GTG TC

15 :HIV.59.1.LLA2C ATT ATC CA[TC] CTT TTA TA[GA] ATT TCT  
CCT ACT TTA GGC ATA GGA CCC GTG TC

:HIV.59.2.LLA2C ATT ATC CA[TC] CTT TTA TA[GA] ATG TCT  
20 CCC ACT TTA GGC ATA GGA CCC GTG TC

:HIV.60.LLA2C CTA TAC AT[TC] CTT ACT ATT TTA TTT  
AAT CCC TTA GGC ATA GGA CCC GTG TC

25 :HIV.62.LLA2C TT[CT] GCA TTT TGG ACC A[AG][CG] AAG  
GTT TCT GTC TTA GGC ATA GGA CCC GTG  
TC

:HIV.63.LLA2C CTC CCT G[AG]C ATG CTG TCA TCA TTT  
30 CTT CTA TTA GGC ATA GGA CCC GTG TC

:HIV.64.1.LLA2C TTC A[TG]T TGG TGT CCT TCC TT[TC] CCA  
CAT TTC TTA GGC ATA GGA CCC GTG TC

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:HIV.64.2.LLA2C TTC A[TG]T TGG TGT CCT TCC CT[TC] CCA  
CAT CTC TTA GGC ATA GGA CCC GTG TC

5 :HIV.65.LLA2C GCC A[GA]A T[CT]T TCC CTA AAA AAT TAG  
CCT GTC TTA GGC ATA GGA CCC GTG TC

:HIV.98.LLA2C [AG]TC CCA [TG]TC TGC AGC TTC CTC ATT  
GAT [GA]GT TTA GGC ATA GGA CCC GTG TC

10 :HIV.66.LLA2C ATC ATT TTT GGT TTC CAT [CT]TT C[CT]T  
GGC AAA TTA GGC ATA GGA CCC GTG TC

:HIV.67.LLA2C TGT C[TC]T ACT TTG ATA AAA CCT CCA  
ATT CCC TTA GGC ATA GGA CCC GTG TC

15 :HIV.70.LLA2C TCT CCA [TC]TT [AG]GT [AG]CT GTC TTT  
TTT CTT TAT TTA GGC ATA GGA CCC GTG  
TC

20 :HIV.71.LLA2C GTA CTG ATA TC[TC] A[AC]T CCC TGG TGT  
[CT]TC ATT TTA GGC ATA GGA CCC GTG TC

:HIV.72.LLA2C GGT GAT CCT TTC CAT CCC TGT GG[ACT]  
AGC ACA TTA GGC ATA GGA CCC GTG TC

25 :HIV.73.LLA2C TAA GAT TTT TGT CAT GCT AC[TA] [TC]TG  
GAA TAT TTA GGC ATA GGA CCC GTG TC

:HIV.69.LLA2C AGA [TC]CC TAC ATA CAA ATC ATC CAT  
GTA TTG TTA GGC ATA GGA CCC GTG TC

30 :HIV.74.LLA2C TAT TTT TG[TC] TCT ATG [CT]TG [CT]CC  
TAT TTC TAA TTA GGC ATA GGA CCC GTG  
TC

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:HIV.75.LLA2C ATG [TC]TT TTT [GA]TC TGG TGT GGT  
AA[GA] TCC CCA TTA GGC ATA GGA CCC  
GTG TC

5 :HIV.76.LLA2C ATA [AC]CC CAT CCA AAG [GA]AA TGG  
[AG]GG TTC TTT TTA GGC ATA GGA CCC  
GTG GTC

:HIV.77.LLA2C TA[CT] TAA GTC TTT TGA TGG GTC ATA  
10 ATA [TC]AC TTA GGC ATA GGA CCC GTG TC

:HIV.78.1.LLA2C TGT TTT CAG ATT TTT AAA TGG [CT]TC  
TTG ATA TTA GGC ATA GGA CCC GTG TC

15 :HIV.78.2.LLA2C TGT TTT CAG ATT TTT ATA TTG [CT]TC  
TTG GTA TTA GGC ATA GGA CCC GTG TC

:HIV.79.LLA2C G[TC]T AA[TC] TGT TT[TC] ACA TCA TTA  
20 GTG TGG GCA TTA GGC ATA GGA CCC GTG  
TC

:HIV.80.LLA2C GGA [GA]T[CT] TTT CCC CAT AT[TC] ACT  
ATG CTT TCT TTA GGC ATA GGA CCC GTG  
TC

25 :HIV.81.LLA2C CCC CAT CTA CAT AGA A[GAC]G TTT CTG  
C[TA]C CTA TTA GGC ATA GGA CCC GTG TC

:HIV.82.LLA2C TGC TTG TAA [CT]TC AGT [TC]TT CTG ATT  
30 TGT TGT TTA GGC ATA GGA CCC GTG TC

:HIV.83.LLA2C ATC TGG TTG TGC TTG AAT [GA]AT [TC]CC  
[TC]AA TGC ATT AGG CAT AGG ACC CGT  
GTC

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:HIV.84.LLA2C ATC TAC TTG TTC ATT TCC TCC AAT  
[TC]CC TTT TTA GGC ATA GGA CCC GTG TC

5 :HIV.85.LLA2C TAG CCA TTG CTC TCC AAT T[AG][CT] TGT  
GAT ATT TTA GGC ATA GGA CCC GTG TC

:HIV.86.LLA2C GAC ATT TAT CAC AGC T[GA]G CTA CTA  
TTT C[TC]T TTA GGC ATA GGA CCC GTG TC

10 :HIV.87.LLA2C TAT [AG]TA [TG]CC ACT GGC TAC ATG  
[AG]AC TGC TAC TTA GGC ATA GGA CCC  
GTG TC

:HIV.88.LLA2C TTT TAC TGG CCA TCT TCC TGC TAA TTT  
15 TAT TAG GCA TAG GAC CCG TGT C

:HIV.89.LLA2C TAC TCC TTG ACT TTG GGG [AG]TT GTA  
GGG AAT TTA GGC ATA GGA CCC GTG TC

20 :HIV.90.LLA2C TCT [TC]TC CCC TGC ACT GTA [CT]CC CCC  
AAT CCC TTA GGC ATA GGA CCC GTG TC

:HIV.91.LLA2C TAG TTT GTA TGT CTG TTG CTA T[TC]A  
TG[TC] CTA TTA GGC ATA GGA CCC GTG TC  
25

:HIV.92.LLA2C TTT GAA TTT TTG T[AG]A TTT G[TC]T TTT  
GTA [AG]TT TTA GGC ATA GGA CCC GTG TC

:HIV.93.LLA2C TCC AGA G[GTA]A G[CT]T TTG CTG GTC  
30 CTT TCC AAA TTA GGC ATA GGA CCC GTG  
TC

:HIV.94.LLA2C TAT T[AG]T C[TC]T GTA TTA CTA CTG CCC  
CTT CAC TTA GGC ATA GGA CCC GTG TC  
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:HIV.95.1.LLA2C      TT[GA] CTT TTC TTC TTG GCA CTA CTT  
TTA T[GA]T TTA GGC ATA GGA CCC GTG TC

5      :HIV.95.2.LLA2C      TT[GA] CTT TTC TTC TTG GTA CTA CCT  
TTA T[GA]T TTA GGC ATA GGA CCC GTG TC

:HIV.103.LLA2C      TTT TCT TTT AAA ATT GTG [AG]AT GAA  
[TC]AC TGC TTA GGC ATA GGA CCC GTG TC

10      D.      Comparison of the pII template probes in  
the assay of HIV in human plasma.

Samples of normal human plasma with varying amounts of a synthetic HIV target sequence were prepared as described in Example 2. 10  $\mu$ l of plasma were added to  
15 12.5  $\mu$ l of extraction buffer (10mM Tris pH 8.0, 150 mM NaCl, 10 mM EDTA, 1% SDS 40 $\mu$ g/ml sonicated salmon sperm DNA, and 2mg/ml proteinase K) and incubated in wells of a microtiter dish at 65°C for 30 minutes. The wells were first prepared by binding a single stranded  
20 oligonucleotide with a defined sequence to the solid substrate as described in Example 2. 5 $\mu$ l of 1N NaOH with 12.5 fmoles of HIV capture and linker probes/well (described above) were added and the mixture was further incubated at 65°C for 30 minutes. Next 13  $\mu$ l of MOPS-  
25 SSC (0.77 M 3-[N-morpholino]propanesulfonic acid, 1.845 M NaCl, 0.185 M Na Citrate) were added and the mixture further incubated at 65°C for 2 hours.

Wells were then washed 2 times with wash buffer A (0.1 X SSC, 0.1% SDS). Following the washes, 30 fmoles  
30 of T7 template probe in 40  $\mu$ l horse hyb mix (50% horse serum, 0.6 M NaCl, 0.06 M Na Citrate, .1% SDS) were added and the mixture incubated at 55°C for 1 hour. (The horse hybridization mix was prepared as follows: for ten ml - 504  $\mu$ l water (treated with diethyl pyrocarbonate,  
35 DEPC), 336  $\mu$ l 10% SDS, 60  $\mu$ l 1 M Tris HCl (pH8), 100 $\mu$ l of

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25mg/ml proteinase K, 5 ml horse serum, incubated 65°C for 2 hours, add 1 ml water (DEPC treated) and 2 ml 20X SSC).

The wells were then washed 2 times with wash buffer A and 2 times with wash buffer B (0.1X SSC). Next 40 µl of transcription mix (40 mM Tris-HCl pH 8, 20 mM MgCl<sub>2</sub>, 80 units T7 polymerase (New England Biolabs), 10mM DTT, 0.15 mg/ml BSA, 1.25 mM each of ATP, UTP, GTP, and CTP, 1600 units/ml RNasin) were added and the mixture incubated in a 37 degree oven for 1.5 hours.

New wells were again prepared by binding single stranded oligonucleotide with a defined sequence to the solid substrate (described in Example 2). To the new well was added 12.5 µl of extraction buffer (2mg/ml proteinase K), 5µl of proteinase K/SDS treated human serum, 15µl 20X SSC, 5 µl 10% SDS containing 12.5 fmoles of transcript capture and amplifier linker probes, and 12.5 µl of the mixture from the first well which contains the newly formed RNA transcripts. This mixture was incubated 65°C for 2 hours.

Note that, in contrast to the protocol of Example 2, transcript capture and amplifier linker probes are used to sandwich the RNA transcript (Figure 5). These probes serve as bridges between the transcript and the immobilized nucleotide on the one hand, and between the transcript and the amplifier probe (to be added) on the other hand.

The wells are next washed 2 times with wash buffer A. Forty µl of horse hybridization mix containing 100 fmoles of comb-like amplifier probe (as in Example 2) added to each well and incubated at 55°C for 15 minutes. Wells were then washed 2X with wash buffer A. Forty µl of horse hybridization mix containing 100 fmoles of alkaline phosphatase probe is added to each well and incubated at 55°C for 15 minutes. (The horse

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hybridization mixture is pretreated to remove residual RNase activity according to the preparation protocol described above except that, after the 65 degree incubation, the solution is cooled and 60 $\mu$ l of 100 mM phenyl-methyl sulfonyl fluoride (PMSF) is added to inactivate the proteinase K, and the mixture is further incubated at 37°C for 1 hour).

The samples were assayed by alkaline phosphatase detection as described in Example 2 above. The wells were washed twice with wash buffer A, twice with wash buffer B. 20 $\mu$ l of dioxetane reagent were added and incubated at 37°C for 30 minutes and the wells were then read in a luminometer.

#### E. Results

Panels of various amounts of cloned HIV DNA added to samples of human serum were prepared and assayed as described above. Each panel was assayed using one of the three PII T7-template probes described above. The sensitivity and amplification are shown in Figure 6. As a control standard, a panel of HIV DNA was assayed using only the comb-like signal amplification probe assay described in E.P. Pub. 0317077. The use of the T7 template probe provides an approximate 30 fold increase in sensitivity and an approximate 50 fold increase in amplification relative to the non-polymerase standard.

#### Example 10

##### Hybridization Assay for Hepatitis C Virus (HCV) DNA Using the Microtiter Dish Assay Procedure and T7 RNA Polymerase

Assays for the presence of HCV-specific DNA and RNA are performed as described in Example 9. Sets of template linker and analyte capture probes are prepared as described in Example 2 and designed to hybridize to

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portions of the HCV genome. These probes are disclosed in the commonly owned PCT US90/02853, filed 18 May 1990. Each probe of the set is a 50-mer in which the first 30 residues at the 5'-end are complementary to HCV sequences and the remaining residues are the system -specific analyte capture and template linker sequences described in Example 2.

In the same manner, other pathogenic bacterial, viral and parasitic strains and antibiotic resistance-conferring genes can be screened. It will be appreciated that the invention assay may be adapted to conduct multiple assays for different analytes simultaneously. In one format, by changing the label and the amplifier probe sequences for a new analyte (as well as the analyte specific sequences in the analyte capture and template linker probes) it is possible to detect two different analytes in the same sample on the same solid phase. Alternatively, by synthesizing different oligonucleotides bound to the solid support (Example 2B, supra) for each analyte, and attaching each bound oligonucleotide sequence to different positions on a membrane strip, it is possible to perform several different assays simultaneously with the same label.

Modifications of the above-described modes for carrying out the invention that are obvious to those of skill in nucleic acid chemistry, biochemical and clinical assays and related fields are intended to be within the scope of the following claims.

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We claim:

1. A polydeoxynucleotide construct for use as  
a signal amplifier in hybridization assays comprising  
5 three domains:
  - (a) a first domain (A) which is single-stranded  
and has a nucleotide sequence complementary to a target  
sequence;
  - (b) a second domain (B) which is double-  
10 stranded and capable of function as a promoter for a  
DNA-dependent RNA polymerase enzyme activity; and
  - (c) a third domain (C) which is either single-  
or double-stranded and adjacent to said second domain,  
such that said third domain is capable of functioning as  
15 a template for the promoter activity of said second  
domain.
2. The polydeoxynucleotide construct of  
claim 1 in which said DNA-dependent RNA polymerase  
20 activity is derived from the bacteriophage T7.
3. The polydeoxynucleotide construct of  
claim 1 in which said DNA-dependent RNA polymerase  
activity is derived from the bacteriophage T3.  
25
4. The polydeoxynucleotide construct of  
claim 1 in which said DNA-dependent RNA polymerase  
activity is derived from the bacteriophage SP6.
- 30 5. The polydeoxynucleotide construct of  
claim 1 in which said first domain A is at least 10 and  
no more than 40 nucleotides long.

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6. The polydeoxynucleotide construct of claim 1 in which said first domain A is at least 15 and no more than 30 nucleotides long.

5           7. The polydeoxynucleotide construct of claim 5 in which said second domain B is at least 12 and no more than 40 nucleotides long.

8. The polydeoxynucleotide construct of claim 5 in which said second domain B is at least 17 and no more than 30 nucleotides long.

9. The polydeoxynucleotide construct of claim 7 in which said third domain C is at least 30 and no more than 10000 nucleotides long.

10           10. The polydeoxynucleotide construct of claim 7 in which said third domain C is at least 40 and no more than 80 nucleotides long.

20           11. The polydeoxynucleotide construct of claim 7 in which said third domain C is at least 2 Kb and no more than 10 Kb in length.

25           12. The polydeoxynucleotide construct of claim 7 in which said third domain C is at least 3 Kb and no more than 4 Kb in length.

30           13. The polydeoxynucleotide construct of claim 7 in which said third domain C is comprised of substantially all of the genome of Hepatitis B virus.

14. The polydeoxynucleotide construct of claim 2 in which the sequence for said second domain B comprises the sequence :

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5'-TAA TAC GAC TCA CTA TA-3'

3'-ATT ATG CTG AGT GAT AT-5'

5           15. The polydeoxynucleotide construct of  
claim 2 in which the nucleotide sequence of said second  
domain B is:

5'-CTG GCT TAT CGA AAT TAA TAC GAC TCA CTA TA-3'  
10       3'-GAC CGA ATA GCT TTA ATT ATG CTG AGT GAT AT-5'.

16. The polydeoxynucleotide construct of  
claim 1 in which the 5' residue of the upper strand of  
the nucleotide sequence for said third domain C, is  
15 adjacent to said second domain B, and is a guanosine  
residue.

17. The polydeoxynucleotide construct of  
claim 1 in which the sequence for said third domain C is:  
20

5'-GGG AGA TGT GGT TGT CGT ACT TAG CGA AAT ACT GTC CGA  
GTC G-3'  
3'-CCC TCT ACA CCA ACA GCA TGA ATC GCT TTA TGA CAG GCT  
CAG C-5'

25

18. The polydeoxynucleotide constructs of  
claim 17 in which the 3' end of the upper strand of the  
DNA nucleotide sequence of said second domain B is  
attached to the 5' end of the upper strand of said  
30 nucleotide sequence of domain C.

19. The polydeoxynucleotide construct of  
claim 1 in which the transcript of said third domain C  
has two subdomains:

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(a) a first subdomain,  $c_1$ , which is capable of hybridizing to an oligonucleotide capture linker, said capture linker being capable of hybridizing to a polynucleotide immobilized on a solid substrate; and

(b) a second subdomain,  $c_2$ , which is capable of binding to an oligonucleotide amplifier linker, said amplifier linker capable of binding to a quantifiable probe.

20. The polydeoxynucleotide construct of claim 1 in which said first and second domains, B and C, are present in multiple repeating units.

21. A method of amplifying a biological signal used to detect and quantify an oligonucleotide analyte in a hybridization assay comprising:

(i) immobilizing said analyte, directly or indirectly, on a solid substrate; and

hybridizing the polydeoxynucleotide construct of claim 1, directly or indirectly to the analyte;

(ii) removing unhybridized polydeoxynucleotide constructs;

(iii) transcribing multiple copies of RNA oligomers which are complementary to the template sequence,  $c'$ , of said third domain, C, of said polydeoxynucleotide construct via a DNA-dependent RNA polymerase activity; and

(iv) detecting the amount of RNA transcripts formed in step (iii).

22. The nucleic acid hybridization assay of claim 21 in which said second domain, B, is derived from the DNA-dependent RNA polymerase of the bacteriophage T7.



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23. The nucleic acid hybridization assay of claim 21 in which said second domain, B, is derived from the DNA-dependent RNA polymerase of the bacteriophage T3.

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24. The nucleic acid hybridization assay of claim 21 in which said second domain, B, is derived from the DNA-dependent RNA polymerase of the bacteriophage SP6.

10

25. The nucleic acid hybridization assay of claim 21 in which said polydeoxynucleotide construct is hybridized directly to a single-stranded analyte.

15

26. The nucleic acid hybridization assay of claim 21 wherein said polydeoxynucleotide construct is hybridized to an oligonucleotide linker, said linker having a domain which is capable of forming stable hybrids with the analyte.

20

27. The nucleic acid hybridization assay of claim 21 in which:

25

(a) said third domain, C, of said polydeoxynucleotide construct is transcribed in the presence of labeled ribonucleotide triphosphates;

30

(b) the transcripts of said third domain have a first subdomain,  $c_1$ , complementary to a oligonucleotide capture probe which is immobilized on a solid substrate; and

(c) said transcripts are immobilized and quantified.

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28. The nucleic acid hybridization assay of claim 21 in which:

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(a) said third domain, C, of said polydeoxy-nucleotide construct is transcribed in the presence of biotinylated ribonucleotides triphosphates;

5 (b) said transcript of said third domain has a first subdomain,  $c_2$ , which is complementary to a labeled oligonucleotide probe; and

(c) said transcripts are immobilized upon an avidinylated solid substrate; and

10 (d) said transcripts are quantified.

29. The nucleic acid hybridization assay of claim 21 in which:

15 (a) said third domain, C, of said polydeoxynucleotide construct is transcribed in the presence of both labeled and biotinylated ribonucleotide triphosphates;

(b) said transcripts are immobilized upon an avidinylated solid substrate; and

20 (c) said transcripts are quantified.

25

30

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(b) said transcript is hybridized to said transcript capture probe and to said linker probe to form a transcript sandwich;

5 (c) said transcript sandwich is hybridized to an oligonucleotide immobilized on a solid substrate;

(d) said immobilized sandwich is hybridized to a labelling oligonucleotide; and

10 (e) said transcripts are quantified.

32. The nucleic acid hybridization assay of claim 21 in which:

15 (a) the transcript of said third domain, C, of said polydeoxynucleotide construct has two subdomains:

(i) a first subdomain,  $c_1$ , which is complementary to a transcript capture probe, said transcript capture probe being capable of hybridizing to an oligonucleotide which has been immobilized on a solid substrate; and  
20 (ii) a second subdomain,  $c_2$ , which is complementary to an amplifier linker probe, said linker probe being  
25 capable of hybridizing to an amplifier probe;

(b) said transcript is hybridized to said transcript capture probe and to said amplifier linker probe to form a transcript sandwich;

30 (c) said transcript sandwich is hybridized to an oligonucleotide immobilized on a solid substrate;

(d) said immobilized sandwich is hybridized to an amplifier probe;

35

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30. The nucleic acid hybridization assay of claim 21 in which:

(a) the transcript of said third domain, C, of said polydeoxynucleotide construct has two subdomains:

- (i) a first subdomain,  $c_1$ , which is complementary to an oligonucleotide capture probe, said probe being immobilized on a solid substrate; and
- (ii) a second subdomain,  $c_2$ , which is complementary to a labeled oligonucleotide probe;

(b) said transcript is hybridized to said capture probe;

(c) said labeled probe is hybridized to said transcripts; and,

(d) said transcripts are quantified.

31. The nucleic acid hybridization assay of claim 21 in which:

(a) the transcript of said third domain, C, of said polydeoxynucleotide construct has two subdomains:

- (i) a first subdomain,  $c_1$ , which is complementary to a transcript capture probe, said transcript capture probe being capable of hybridizing to an oligonucleotide which has been immobilized on a solid substrate; and
- (ii) a second subdomain,  $c_2$ , which is complementary to a linker probe, said linker probe being capable of hybridizing to a labeling oligonucleotid ;

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- (e) said amplifier probe is hybridized to a labeling oligonucleotide; and  
(e) said transcripts are quantified.

5

33. The nucleic acid hybridization assay of claim 32 in which the RNA transcript is transcribed from DNA of Hepatitis B Virus.

10

34. The nucleic acid hybridization assay of claim 21 in which said second and third domains, B and C, of said polydeoxynucleotide construct are present in multiple repeating units.

15

35. The method of claim 21 used to detect the presence of N. gonorrhoeae in a biological sample in which the analyte comprises a DNA or RNA segment of N. gonorrhoeae.

20

36. The method of claim 21 used to detect the presence of Hepatitis B virus in a biological sample in which the analyte comprises a DNA or RNA segment of Hepatitis B virus.

25

37. The method of claim 21 used to detect the presence of bacteria containing the beta-Lactamase TEM-1 gene, in a biological sample in which the analyte comprises a DNA or RNA segment of the beta-Lactamase TEM-1 gene.

30

38. The method of claim 21 used to detect the presence of Chlamydia in a biological sample in which the analyte comprises a DNA or RNA segment of Chlamydia.

35

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39. The method of claim 21 used to detect the presence of bacteria containing the tet M determinant in a biological sample in which the analyte comprises a DNA or RNA segment of the tet M determinant.

5

40. The method of claim 21 used to detect the presence of HIV in a biological sample in which the analyte comprises a DNA or RNA segment of HIV.

10

41. The method of claim 21 used to detect the presence of HCV in a biological sample in which the analyte comprises a DNA or RNA segment of HCV.

42. A method of amplifying the biological signal used to detect and quantify a ligand receptor in a hybridization assay comprising the following steps:

(a) immobilizing said ligand receptor directly or indirectly on a solid phase;

(b) binding to the ligand receptor a ligand specific for said receptor, said ligand being coupled to an oligonucleotide complementary to the first domain of the construct of claim 1;

(c) removing unhybridized ligand;

(d) transcribing multiple copies of RNA oligomers which are complementary to the template sequence, c', of the third domain, C, of said polydeoxynucleotide construct via a DNA-dependent RNA polymerase activity; and

(e) quantifying the RNA transcripts.

30

43. The hybridization assay of claim 42 in which the ligand receptor is an antigen and the ligand is an antibody which immunologically reacts with the antigen.

35

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44. A template linker probe specific for a DNA or RNA analyte substantially similar to HBV genomic DNA.

45. The template linker probe of claim 44  
5 wherein said linker probe has the DNA sequence: TGA CTG  
[CG]CG ATT GGT [GA]GA GGC AGG [AC]GG AGG TTA GGC ATA GGA  
CCC GTG TC.

46. The template linker probe of claim 44  
10 wherein said linker probe has the DNA sequence: CTT  
G[AT][CT] GGG [GA]TT GAA GTC CCA ATC TGG ATT TTA GGC ATA  
GGA CCC GTG TC.

47. The template linker probe of claim 44  
15 wherein said linker probe has the DNA sequence: GTT GCG  
TCA GCA AAC ACT TGG CA[CG] AGA CC[AT] TTA GGC ATA GGA CCC  
GTG TC.

48. The template linker probe of claim 44  
20 wherein said linker probe has the DNA sequence: TAA GTT  
GGC GAG AAA GT[GA] AAA GCC TG[TC] TT[AC] TTA GGC ATA GGA  
CCC GTG TC.

49. The template linker probe of claim 44  
25 wherein said linker probe has the DNA sequence: GCA GCA  
AA[GA] CCC AAA AGA CCC ACA A[TG][TA] C[TG][TC] TTA GGC  
ATA GGA CCC GTG TC.

50. The template linker probe of claim 44  
30 wherein said linker probe has the DNA sequence: ATG TAT  
ACC CA[GA] AGA CA[AG] AAG AAA ATT GGT TTA GGC ATA GGA CCC  
GTG TC.

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51. The template linker probe of claim 44 wherein said linker probe has the DNA sequence: TAG AGG ACA AAC GGG CAA CAT ACC TTG [AG]TA TTA GGC ATA GGA CCC GTG TC.

5

52. The template linker probe of claim 44 wherein said linker probe has the DNA sequence: GAT GAG GCA TAG CAG CAG GAT GAA GAG GAA TTA GGC ATA GGA CCC GTG TC.

10

53. The template linker probe of claim 44 wherein said linker probe has the DNA sequence: GAT AAA ACG CCG CAG ACA CAT CCA GCG ATA TTA GGC ATA GGA CCC GTG TC.

15

54. The template linker probe of claim 44 wherein said linker probe has the DNA sequence: GGA CAA [AG]TT GGA GGA CA[GA] GAG GTT GGT GAG TTA GGC ATA GGA CCC GTG TC.

20

55. The template linker probe of claim 44 wherein said linker probe has the DNA sequence: TTG GAG GTT GGG GAC TGC GAA TTT TGG CCA TTA GGC ATA GGA CCC GTG TC.

25

56. The template linker probe of claim 44 wherein said linker probe has the DNA sequence: CCA CCA CGA GTC TAG ACT CTG [CT]GG TAT TGT TTA GGC ATA GGA CCC GTG TC.

30

57. The template linker probe of claim 44 wherein said linker probe has the DNA sequence: GAT TCT TGT CAA CAA GAA AAA CCC CGC CTG TTA GGC ATA GGA CCC GTG TC.

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58. The template linker probe of claim 44 wherein said linker probe has the DNA sequence: CAC GAG [CA]AG GGG TCC TAG GAA TCC TGA TGT TTA GGC ATA GGA CCC GTG TC.

5

59. The template linker probe of claim 44 wherein said linker probe has the DNA sequence: CAG GGT TTA CTG TTC C[ TG]G AAC TGG AGC CAC TTA GGC ATA GGA CCC GTG TC.

10

60. An analyte capture probe specific for a DNA or RNA analyte substantially similar to HBV genomic DNA.

15

61. The analyte capture probe of claim 60 wherein said capture probe has the DNA sequence: CTT GGC CCC CAA TAC CAC ATC ATC CAT ATA CTT CTT TGG AGA AAG TGG TG.

20

62. The analyte capture probe of claim 60 wherein said capture probe has the DNA sequence: GAA AGC CAA ACA GTG GGG GAA AGC CCT ACG CTT CTT TGG AGA AAG TGG TG.

25

63. The analyte capture probe of claim 60 wherein said capture probe has the DNA sequence: CAC TGA ACA AAT GGC ACT AGT AAA CTG AGC CTT CTT TGG AGA AAG TGG TG.

30

64. The analyte capture probe of claim 60 wherein said capture probe has the DNA sequence: GAG AAA CGG [AG]CT GAG GCC C[AC]C TCC CAT AGG CTT CTT TGG AGA AAG TGG TG.

35

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65. The analyte capture probe of claim 60 wherein said capture probe has the DNA sequence: [GC]CG AAA GCC CAG GA[CT] GAT GGG ATG GGA ATA CTT CTT TGG AGA AAG TGG TG.

5

66. An analyte capture probe specific for a DNA or RNA analyte substantially similar to HIV genomic RNA.

10

67. The analyte capture probe of claim 66 wherein said capture probe the DNA sequence: TTC TAC TAC TTT [TC]AC CCA TGC [AG]TT TAA AGC TTC TTT GGA GAA AGT GGTG.

15

68. The analyte capture probe of claim 66 wherein said capture probe has the DNA sequence: TTC TAT TAC TTT [TC]AC CCA TGC [AG]TT CAA AGC TTC TTT GGA GAA AGT GGT G.

20

69. The analyte capture probe of claim 66 wherein said capture probe has the DNA sequence: TGC TTG ATG TCC CCC CAC TGT GTT TAG CAT CTT CTT TGG AGA AAG TGG TG.

25

70. The analyte capture probe of claim 66 wherein said capture probe has the DNA sequence: TGC CTG GTG TCC TCC AAC TAT GTT CAG CAT CTT CTT TGG AGA AAG TGG TG.

30

71. The analyte capture probe of claim 66 wherein said capture probe has the DNA sequence: AGG TGA TAT GGC [CT]TG ATG TA[CT] CAT TTG CCC CTT CTT TGG AGA AAG TGG TG.

35

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72. The analyte capture probe of claim 66 wherein said capture probe has the DNA sequence: CAT GGG TAT [TC]AC TTC TGG GCT [GA]AA [AG]GC CTT CTT CTT TGG AGA AAG TGG TG.

5

73. The analyte capture probe of claim 66 wherein said capture probe has the DNA sequence: TTG [TC]GG GGT GGC [TC]CC [TC]TC TGA TAA TGC TGA CTT CTT TGG AGA AAG TGG TG.

10

74. The analyte capture probe of claim 66 wherein said capture probe has the DNA sequence: AAT TTT T[GA]A AAT TTT [TC]CC TTC CTT TTC CAT CTT CTT TGG AGA AAG TGG TG.

15

75. The analyte capture probe of claim 66 wherein said capture probe has the DNA sequence: AAC TCT T[GA]A AAT TTT [TC]CC TTC CTT TTC CAT CTT CTT TGG AGA AAG TGG TG.

20

76. The analyte capture probe of claim 66 wherein said capture probe has the DNA sequence: TTA CTG GTA CAG T[TC]T CAA TAG G[AG]C TAA T[GT]G CTT CTT TGG AGA AAG TGG TG.

25

77. The analyte capture probe of claim 66 wherein said capture probe has the DNA sequence: TAA C[TC][TC] TTG GGC CAT CCA T[TC]C CTG GCT TTC TTC TTT GGA GAA AGT GGT G.

30

78. The analyte capture probe of claim 66 wherein said capture probe has the DNA sequence: CTT TTA TTT TTT CTT CTG TCA ATG GCC ATC TTC TTT GGA GAA AGT GGT G.

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79. The analyte capture probe of claim 66 wherein said capture probe has the DNA sequence: AAA TAC TGG AGT ATT GTA TGG ATT [CT]TC AGC TTC TTT GGA GAA AGT GGT G.

5

80. A template linker probe specific for a DNA or RNA analyte substantially similar to HIV genomic RNA.

81. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TCC [CG]CC GCT TAA TAC [TC]GA CGC TCT CGC ACC TTA GGC ATA GGA CCC GTG TC.

82. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TTA [TA]AT AAT GAT [CT]TA AGT TCT TCT GAT CCT TTA GGC ATA GGA CCC GTG TC.

83. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: ACT TCC [CT]CT TGG TTC TCT CAT [CT]TG [GA]CC TGG TTA GGC ATA GGA CCC GT GTC.

84. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TTC [CT]TG AAG GGT ACT AGT [AG]GT TCC TGC TAT TTA GGC ATA GGA CCC GTG TC.

85. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: GAT AGG TGG ATT A[CT][TG] TGT CAT CCA T[GC]C TAT TTA GGC ATA GGA CCC GTG TC.

35

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86. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: GAT AGG TGG GTT G[TC][TG] TGT CAT CCA T[GC]C TAT TTA GGC ATA GGA CCC GTG TC.

5

87. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: ATT ATC CA[TC] CTT TTA TA[GA] ATT TCT CCT ACT TTA GGC ATA GGA CCC GTG TC.

10

88. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: ATT ATC CA[TC] CTT TTA TA[GA] ATG TCT CCC ACT TTA GGC ATA GGA CCC GTG TC.

15

89. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: CTA TAC AT[TC] CTT ACT ATT TTA TTT AAT CCC TTA GGC ATA GGA CCC GTG TC.

20

90. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TT[CT] GCA TTT TGG ACC A[AG][CG] AAG GTT TCT GTC TTA GGC ATA GGA CCC GTG TC.

25

91. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: CTC CCT G[AG]C ATG CTG TCA TCA TTT CTT CTA TTA GGC ATA GGA CCC GTG TC.

30

92. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TTC A[TG]T TGG TGT CCT TCC TT[TC] CCA CAT TTC TTA GGC ATA GGA CCC GTG TC.

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93. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TTC A[**TG**]T TGG TGT CCT TCC CT[**TC**] CCA CAT CTC TTA GGC ATA GGA CCC GTG TC.

5

94. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: GCC A[**GA**]A T[**CT**]T TCC CTA AAA AAT TAG CCT GTC TTA GGC ATA GGA CCC GTG TC.

10

95. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: [AG]TC CCA [TG]TC TGC AGC TTC CTC ATT GAT [GA]GT TTA GGC ATA GGA CCC GTG TC.

15

96. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: ATC ATT TTT GGT TTC CAT [CT]TT C[CT]T GGC AAA TTA GGC ATA GGA CCC GTG TC.

20

97. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TGT C[TC]T ACT TTG ATA AAA CCT CCA ATT CCC TTA GGC ATA GGA CCC GTG TC.

25

98. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TCT CCA [TC]TT [AG]GT [AG]CT GTC TTT TTT CTT TAT TTA GGC ATA GGA CCC GTG TC.

30

99. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: GTA CTG ATA TC[TC] A[AC]T CCC TGG TGT [CT]TC ATT TTA GGC ATA GGA CCC GTG TC.

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100. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: GGT GAT CCT TTC CAT CCC TGT GG[ACT] AGC ACA TTA GGC ATA GGA CCC GTG TC.

5

101. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TAA GAT TTT TGT CAT GCT AC[TA] [TC]TG GAA TAT TTA GGC ATA GGA CCC GTG TC.

10

102. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: AGA [TC]CC TAC ATA CAA ATC ATC CAT GTA TTG TTA GGC ATA GGA CCC GTG TC.

15

103. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TAT TTT TG[TC] TCT ATG [CT]TG [CT]CC TAT TTC TAA TTA GGC ATA GGA CCC GTG TC.

20

104. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: ATG [TC]TT TTT [GA]TC TGG TGT GGT AA[GA] TCC CCA TTA GGC ATA GGA CCC GTG TC.

25

105. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: ATA [AC]CC CAT CCA AAG [GA]AA TGG [AG]GG TTC TTT TTA GGC ATA GGA CCC GTG GTC.

30

106. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TA[CT] TAA GTC TTT TGA TGG GTC ATA ATA [TC]AC TTA GGC ATA GGA CCC GTG TC.

35

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107. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TGT TTT CAG ATT TTT AAA TGG [CT]TC TTG ATA TTA GGC ATA GGA CCC GTG TC.

5

108. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TGT TTT CAG ATT TTT ATA TTG [CT]TC TTG GTA TTA GGC ATA GGA CCC GTG TC.

10

109. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: G[TC]T AA[TC] TGT TT[TC] ACA TCA TTA GTG TGG GCA TTA GGC ATA GGA CCC GTG TC.

15

110. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: GGA [GA]T[CT] TTT CCC CAT AT[TC] ACT ATG CTT TCT TTA GGC ATA GGA CCC GTG TC.

20

111. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: CCC CAT CTA CAT AGA A[GAC]G TTT CTG C[TA]C CTA TTA GGC ATA GGA CCC GTG TC.

25

112. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TGC TTG TAA [CT]TC AGT [TC]TT CTG ATT TGT TGT TTA GGC ATA GGA CCC GTG TC.

30

113. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: ATC TGG TTG TGC TTG AAT [GA]AT [TC]CC [TC]AA TGC ATT AGG CAT AGG ACC CGT GTC.

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114. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: ATC TAC TTG TTC ATT TCC TCC AAT [TC]CC TTT TTA GGC ATA GGA CCC GTG TC.

5

115. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TAG CCA TTG CTC TCC AAT T[AG][CT] TGT GAT ATT TTA GGC ATA GGA CCC GTG TC.

10

116. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: GAC ATT TAT CAC AGC T[GA]G CTA CTA TTT C[TC]T TTA GGC ATA GGA CCC GTG TC.

15

117. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TAT [AG]TA [TG]CC ACT GGC TAC ATG [AG]AC TGC TAC TTA GGC ATA GGA CCC GTG TC.

20

118. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TTT TAC TGG CCA TCT TCC TGC TAA TTT TAT TAG GCA TAG GAC CCG TGT C.

25

119. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TAC TCC TTG ACT TTG GGG [AG]TT GTA GGG AAT TTA GGC ATA GGA CCC GTG TC.

30

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120. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TCT [TC]TC CCC TGC ACT GTA [CT]CC CCC AAT CCC TTA GGC ATA GGA CCC GTG TC.

5

121. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TAG TTT GTA TGT CTG TTG CTA T[TC]A TG[TC] CTA TTA GGC ATA GGA CCC GTG TC.

10

122. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TTT GAA TTT TTG T[AG]A TTT G[TC]T TTT GTA [AG]TT TTA GGC ATA GGA CCC GTG TC.

15

123. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TCC AGA G[GTA]A G[CT]T TTG CTG GTC CTT TCC AAA TTA GGC ATA GGA CCC GTG TC.

20

124. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TAT T[AG]T C[TC]T GTA TTA CTA CTG CCC CTT CAC TTA GGC ATA GGA CCC GTG TC.

25

125. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TT[GA] CTT TTC TTC TTG GCA CTA CTT TTA T[GA]T TTA GGC ATA GGA CCC GTG TC.

30

126. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TT[GA] CTT TTC TTC TTG GTA CTA CCT TTA T[GA]T TTA GGC ATA GGA CCC GTG TC.

35

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127. The template linker probe of claim 80 wherein said linker probe has the DNA sequence: TTT TCT TTT AAA ATT GTG [AG]AT GAA [TC]AC TGC TTA GGC ATA GGA CCC GTG TC.

5

10

15

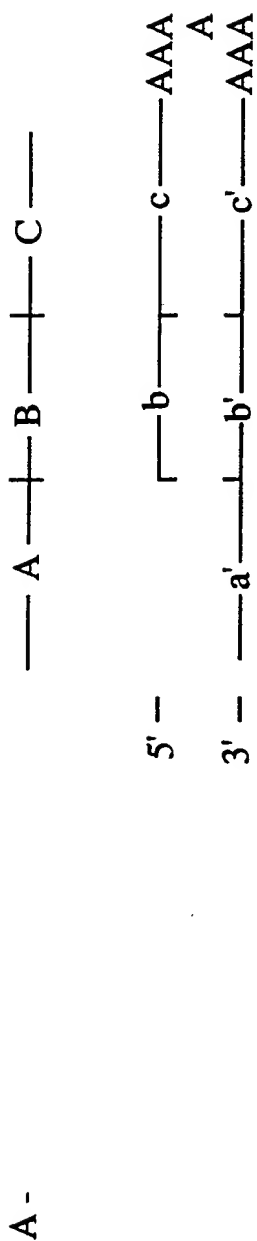
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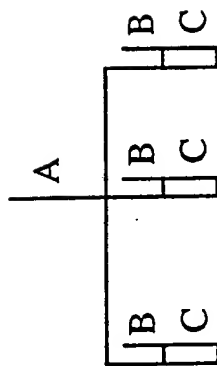
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Figure 1-1



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Figure 1-2

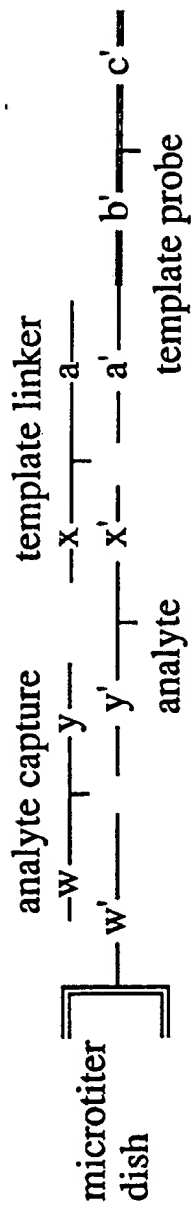


C-

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Figure 2-1

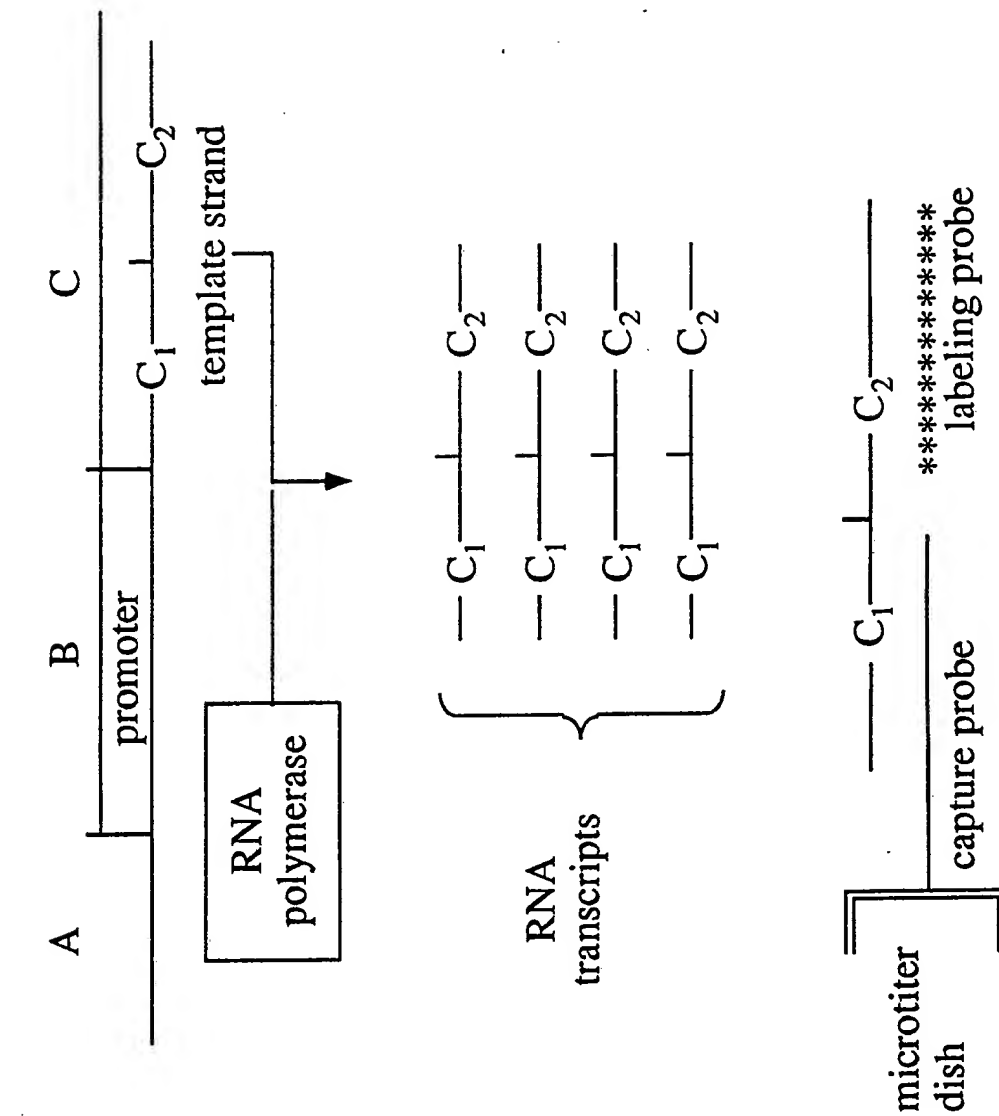


A -

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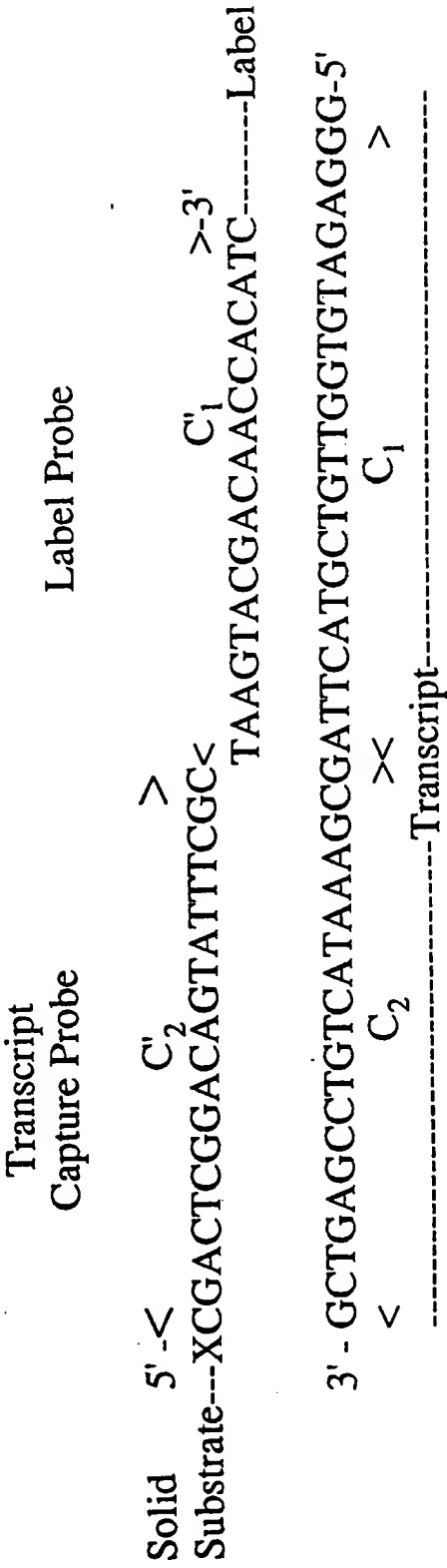
Figure 2-2



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Figure 2-3

C -





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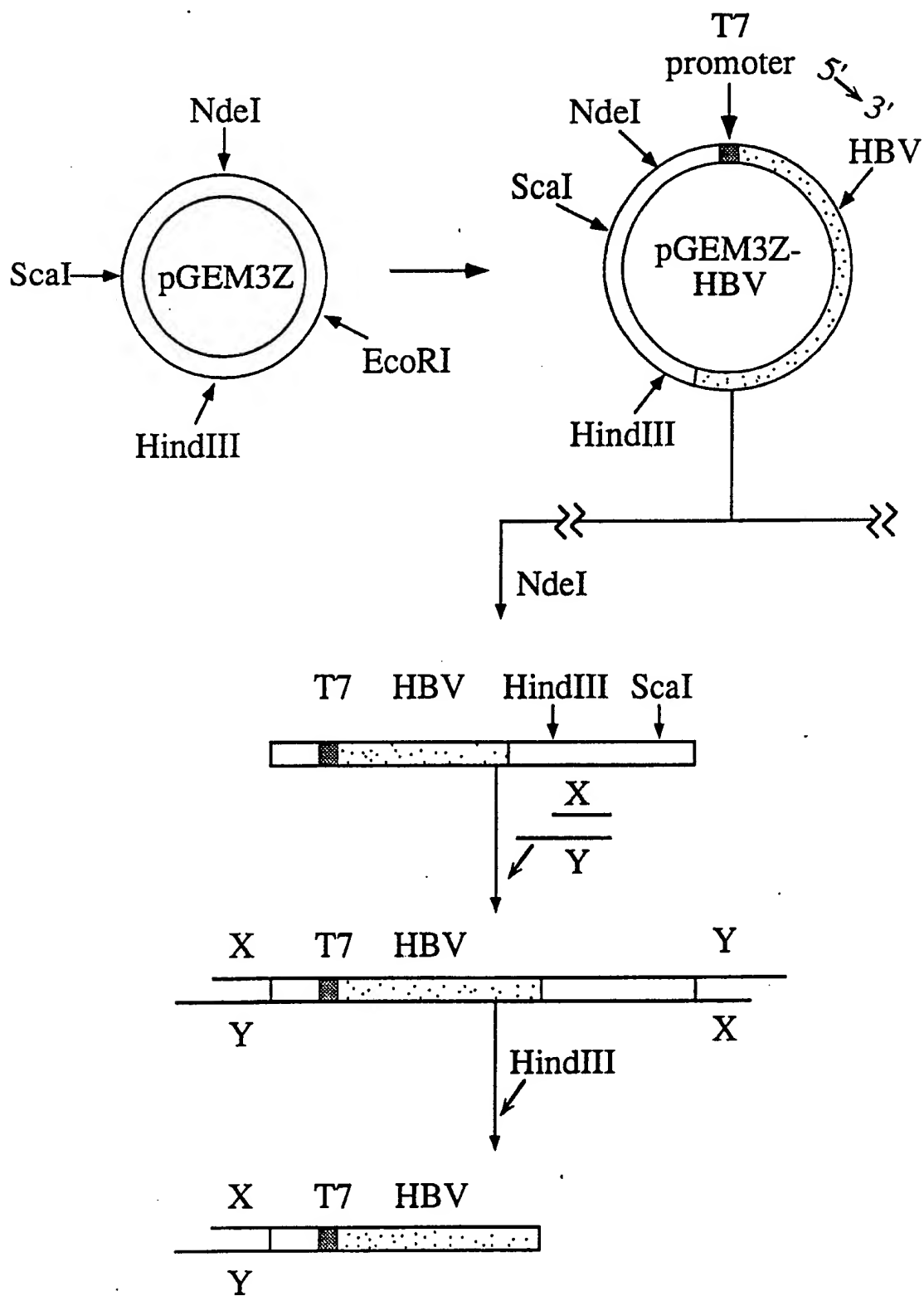


Figure 3A

pII

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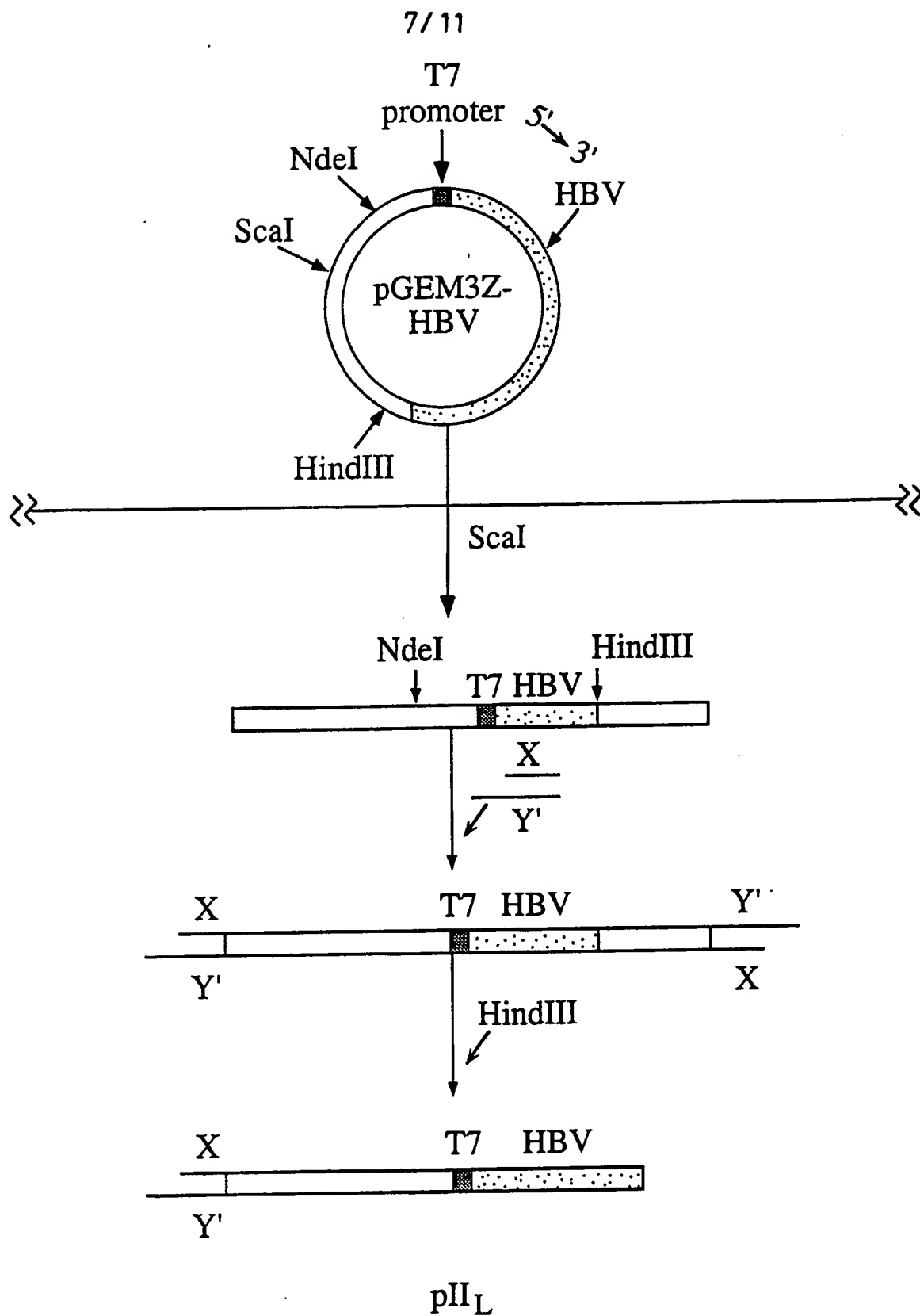


Figure 3B

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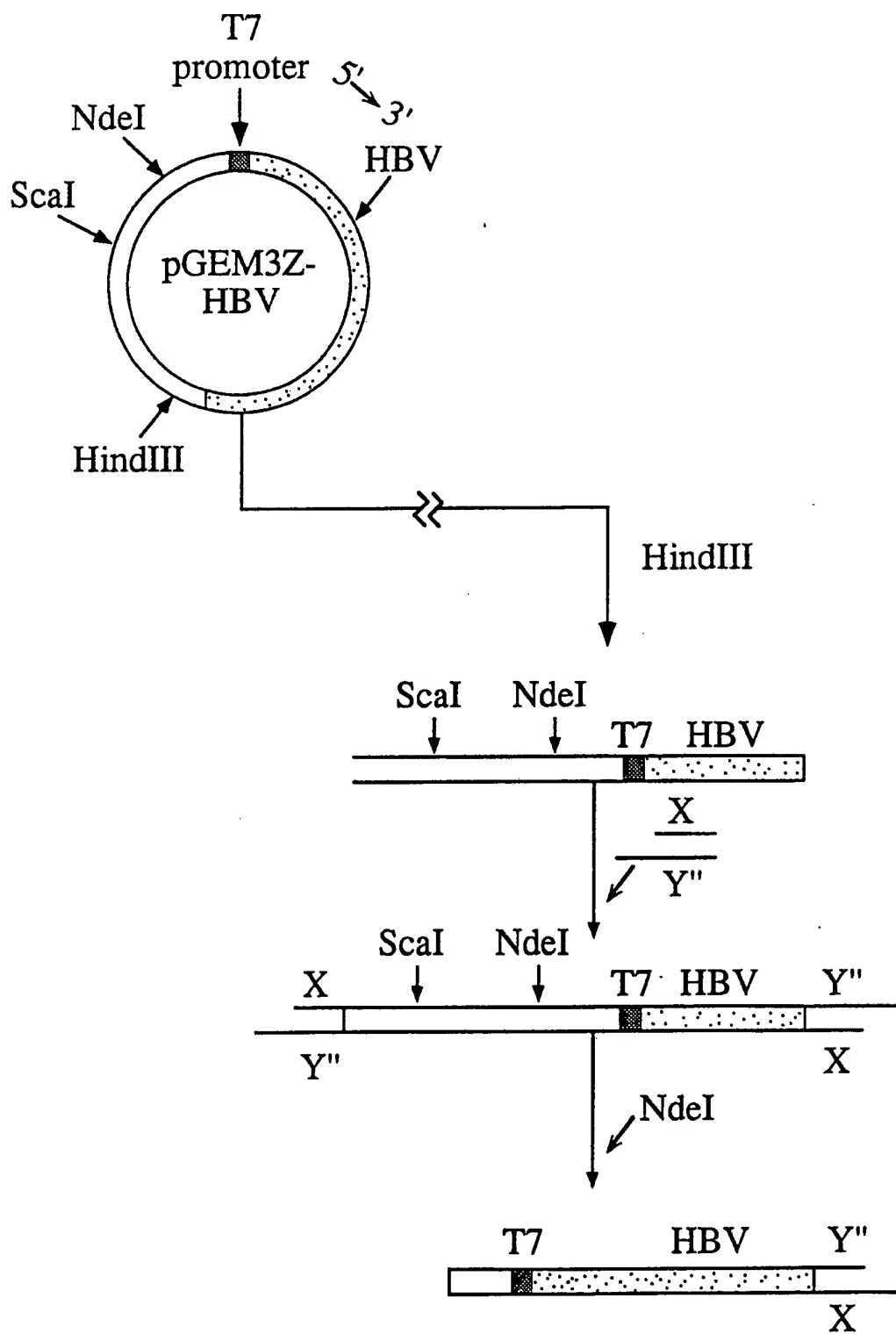
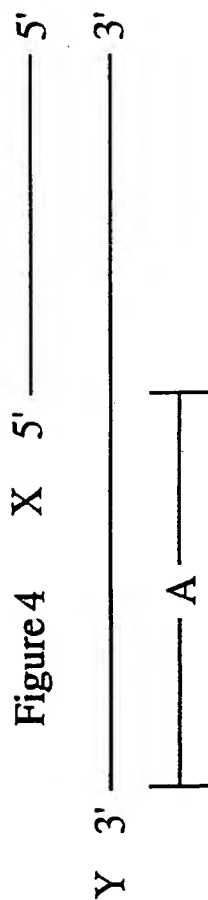


Figure 3C

pII<sub>R</sub>

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OLIGO-N

X - 5'-GGTCGACTAATCGGTAGC

Y - 3'-TCCGTATCCTGGGCACAGCCAGCTGATTAGCCATCGAT

OLIGO-S

X - 5'-GGTCGACTAATCGGTAGC

Y'- 3'-TCCGTATCCTGGGCACAGCCAGCTGATTAGCCATCG

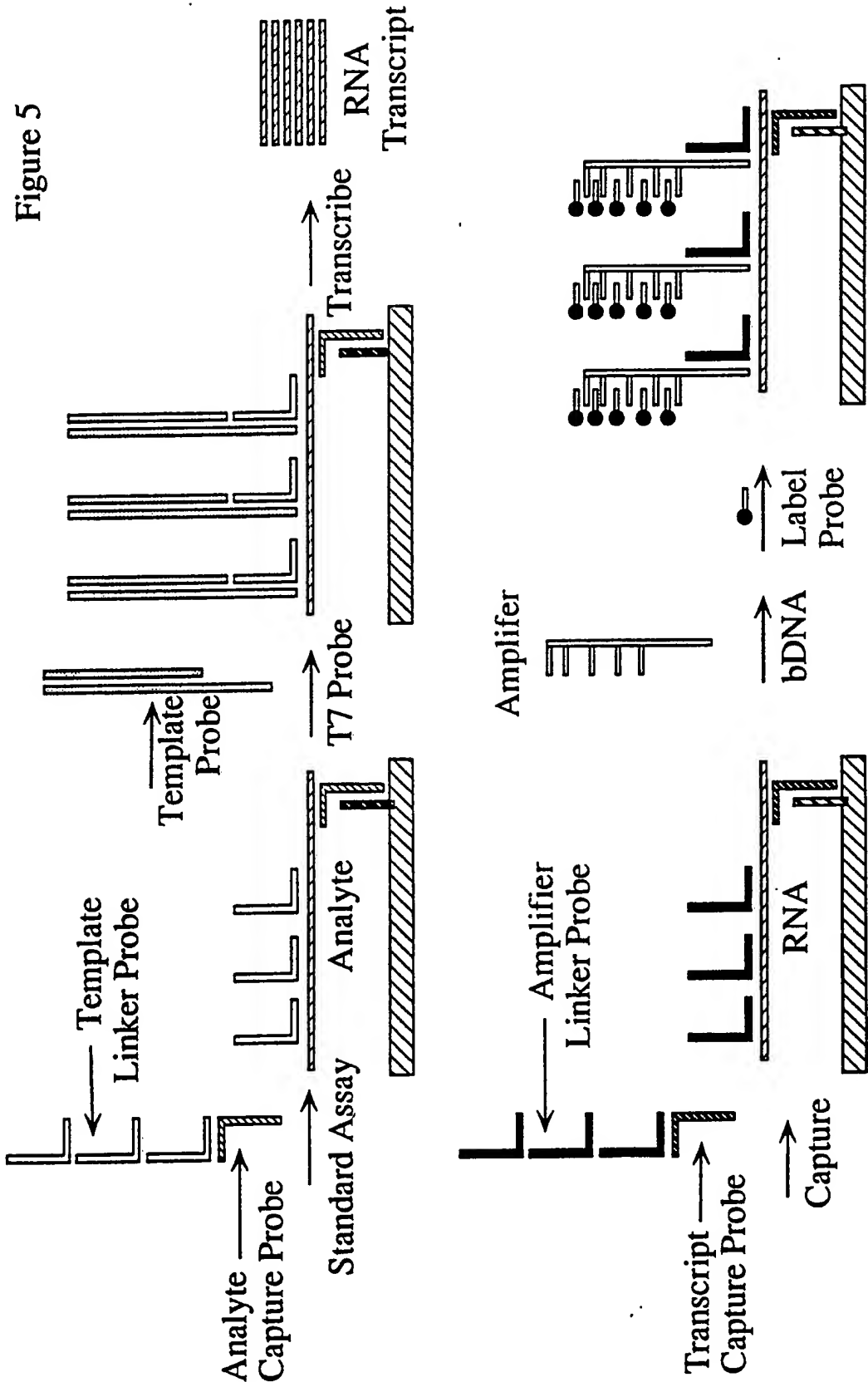
OLIGO-H

X - 5'-GGTCGACTAATCGGTAGC

Y''- 3'-TCCGTATCCTGGGCACAGCCAGCTGATTAGCCATCGTCGA

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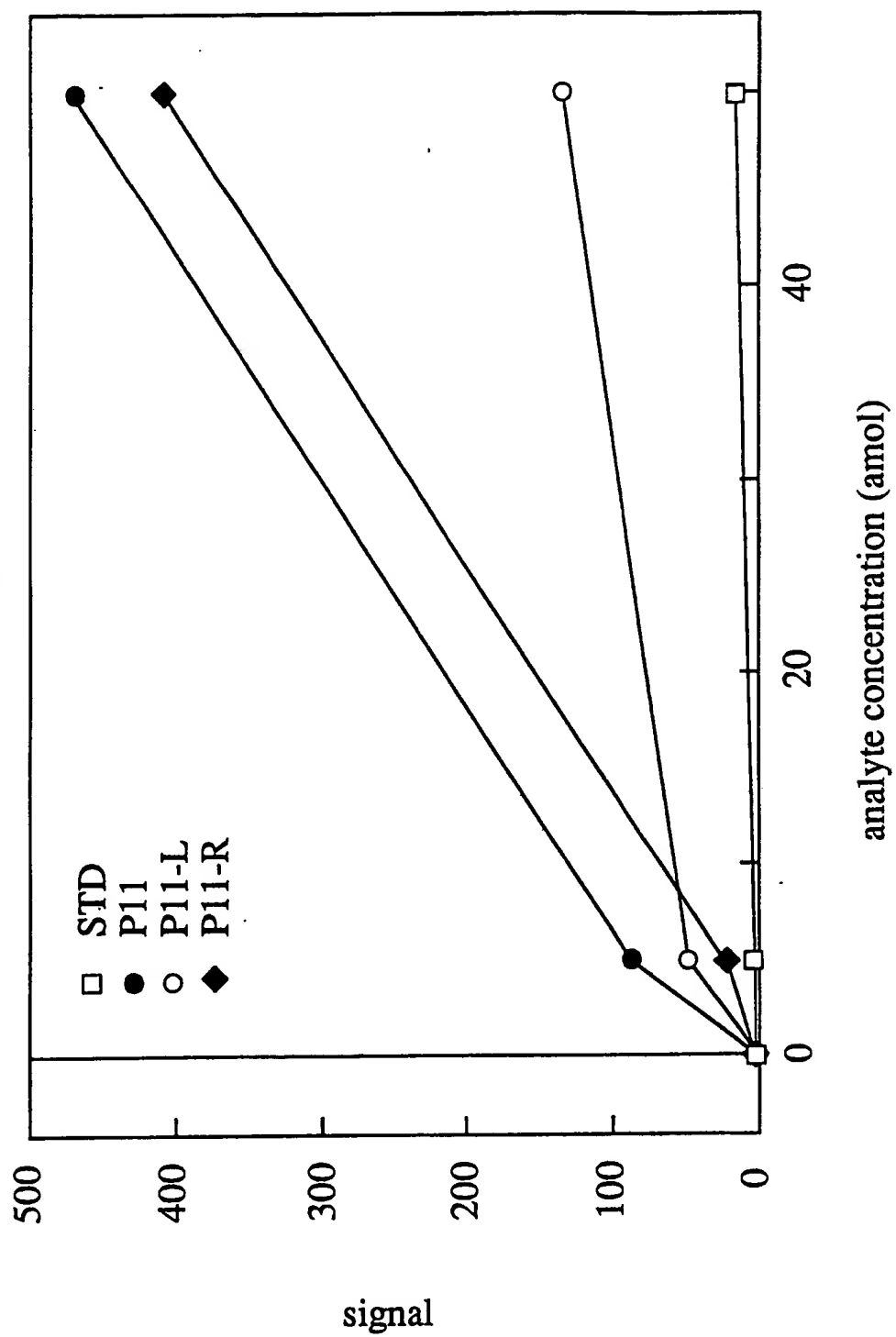
Figure 5



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STD. vs. P11 Probes  
17 Assay

Figure 6



# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/00213

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC I.P.C. (5): C12Q 1/68; C07H 15/12; C12N 15/00 US Cl.: 435/6; 536/27; 935/77, 78														
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border: 1px solid black; text-align: left;">Classification System</th> <th style="border: 1px solid black; text-align: left;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; padding: 5px;">U.S. Cl.</td> <td style="border: 1px solid black; padding: 5px;">435/6; 536/27; 935/77, 78</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div>			Classification System	Classification Symbols	U.S. Cl.	435/6; 536/27; 935/77, 78								
Classification System	Classification Symbols													
U.S. Cl.	435/6; 536/27; 935/77, 78													
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border: 1px solid black; text-align: left;">Category <sup>9</sup></th> <th style="border: 1px solid black; text-align: left;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 15%; border: 1px solid black; text-align: left;">Relevant to Claim No. <sup>13</sup></th> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="border: 1px solid black; padding: 5px;">Proceedings of the National Academy of Sciences, Vol. 77, issued July 1980, Saito et al., "Nucleotide Sequence of the Primary origin of Bacteriophage T7 DNA Replication: Relationship to Adjacent Genes and Regulatory Elements", pages 3917-3921, See entire document.</td> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">1-127</td> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="border: 1px solid black; padding: 5px;">Biochemistry, Vol. 20, issued 1981, Osterman et al, "T7 Ribonucleic Acid Polymerase-Promoter Interaction", pages 4884-4892, see entire documents.</td> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">1-127</td> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="border: 1px solid black; padding: 5px;">Nucleic Acids Research, vol. 16, No. 17, issued 1988, Petty, "A Plasmid Vector for Cloning Directly at the Transcription Initiation Site of a Bacteriophage T7 Promoter", page 8738, see entire document.</td> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">1-127</td> </tr> </table>			Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	X	Proceedings of the National Academy of Sciences, Vol. 77, issued July 1980, Saito et al., "Nucleotide Sequence of the Primary origin of Bacteriophage T7 DNA Replication: Relationship to Adjacent Genes and Regulatory Elements", pages 3917-3921, See entire document.	1-127	X	Biochemistry, Vol. 20, issued 1981, Osterman et al, "T7 Ribonucleic Acid Polymerase-Promoter Interaction", pages 4884-4892, see entire documents.	1-127	X	Nucleic Acids Research, vol. 16, No. 17, issued 1988, Petty, "A Plasmid Vector for Cloning Directly at the Transcription Initiation Site of a Bacteriophage T7 Promoter", page 8738, see entire document.	1-127
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claims or which is cited to establish the prior art (date of publication or other citation or other special reason (is specified))</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p><sup>14</sup> document number of the same patent family</p> </div> </div>														
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black;">Date of Mailing of This International Search Report</td> </tr> <tr> <td style="text-align: center; padding: 5px;">04 March 1991</td> <td style="text-align: center; padding: 5px;">02 MAY 1991</td> </tr> <tr> <td style="border-bottom: 1px solid black;">International Searching Authority</td> <td style="border-bottom: 1px solid black;"> <div style="text-align: center;">  Mindy B. Fleisher </div> </td> </tr> <tr> <td style="text-align: center; padding: 5px;">ISA/US</td> <td></td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of This International Search Report	04 March 1991	02 MAY 1991	International Searching Authority	<div style="text-align: center;">  Mindy B. Fleisher </div>	ISA/US					
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## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Proceedings of the National Academy of Sciences, Vol. 86, issued February 1989, Kwoh et al., "Transcription-Based Amplification system and Detection of Amplified Human Immunodeficiency Virus Type I with a Bead-Based Sandwich Hybridization Format", pages 1173-1177, see the entire document.	1-127
X	US, A, 4,786,600 (Kramer et al.) 22 November 1988, see entire document.	1-127